

**Factors Affecting Oestrogen Action  
in the Human Prostate:  
Does 5 $\alpha$ -Reductase Type 1 Isozyme  
Have a Role?**

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## ABSTRACT OF THESIS

Oestrogens have long been implicated in prostatic pathology including benign prostatic hyperplasia. Their actions and metabolic pathways within the organ are yet unclear. The aim of this project was to investigate the factors that affect the actions of oestrogens in the prostate. A role for 5 $\alpha$ -reductase type 1 isozyme (5 $\alpha$ R1) in oestrogen metabolism was also examined.

Little is known about the functions of 5 $\alpha$ R1 in the human prostate. Substrates for 5 $\alpha$ -reductase are also substrates for aromatase. The hypothesis that 5 $\alpha$ R1 plays a role in the regulation of aromatase activity by limiting substrate availability to the latter in the human prostate was tested in a co-transfection model and in primary cultures of prostatic cells. In COS-1 cells transiently co-transfected with plasmids encoding 5 $\alpha$ R1 and aromatase, inhibition of 5 $\alpha$ R1 led to an increase in aromatase activity but not vice versa. This pattern of interaction between the two enzymes was also shown to occur with endogenous enzymes expressed in primary cultures of prostatic fibroblasts. No aromatase activity was detectable in epithelial cells. By regulating aromatase activity, 5 $\alpha$ R1 may therefore contribute to the local control of oestrogen concentration within the prostate gland.

In this study, prostatic cells were revealed to be oestrogen target cells, not only expressing mRNAs of both subtypes of oestrogen receptor (ER $\alpha$  and ER $\beta$ ) but also responding to oestradiol with increased proliferation rates. This oestrogen stimulation of proliferation was antagonised by the antioestrogen ICI 182,780. Moreover, prostatic fibroblasts aromatised androgen substrates to oestrogens (oestrogen formation). Both fibroblast and epithelial cell cultures also converted oestradiol to oestrone (oestrogen catabolism) but not vice versa. Primary cultures of

prostatic cells therefore responded to oestrogen stimulation and expressed major components for oestrogen action including oestrogen receptors and oestrogen-metabolising enzymes; they would serve as a useful model to further study oestrogen action, regulation and signalling pathways in the prostate.

In addition to the above enzyme inhibitors, the effects of Permixon<sup>®</sup> (a phytotherapeutic agent for the management of prostatic hyperplasia) were investigated. The mechanism of action of Permixon<sup>®</sup> is not entirely clear; however, in previous studies it inhibited both 5 $\alpha$ -reductase isozymes in prostatic cells. Permixon<sup>®</sup> was herein shown to have pro-oestrogenic properties. It not only indirectly increased the conversion of androstenedione to oestrone in fibroblasts by inhibiting 5 $\alpha$ R1, but also inhibited the conversion of oestradiol to the weaker oestrogen oestrone. It is therefore conceivable that Permixon<sup>®</sup> exerts its therapeutic effects by affecting the metabolism of both androgens and oestrogens within the human prostate.

## **DEDICATION**

This thesis is dedicated to the memory of my father with gratitude.



## **ACKNOWLEDGEMENTS**

I am indebted to my supervisor Dr. Fouad Habib, who made this project possible. I wish to thank him for being a gentleman, always supportive and approachable.

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Also, I wish to thank my brother, William, for his moral and financial support, and for believing in me.

Last but not least, I thank Pierre-Fabre Medicament for funding this work.

## **DECLARATION**

I, Clement Kam Man Ho, hereby declare that, unless otherwise specified, the work embodied in this thesis is the result of my own investigation and that this thesis has been composed by myself. This is in accordance with rule 3.8.7 of the University of Edinburgh Postgraduate Study Programme.

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## ABBREVIATIONS

3 $\beta$ Adiol	5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol
5 $\alpha$ A	5 $\alpha$ -androstanedione
5 $\alpha$ R1	5 $\alpha$ -reductase type 1
5 $\alpha$ R2	5 $\alpha$ -reductase type 2
A	androstenedione
A5diol	androst-5-ene-3 $\beta$ ,17 $\beta$ -diol
ATP	adenosine 5' triphosphate
bp	base pairs
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
cDNA	complementary DNA
DCC	dextran coated charcoal
DCC-FCS	dextran coated charcoal-stripped fetal calf serum
DEPC	diethyl pyrocarbonate
DHEA	dehydroepiandrosterone
DHT	5 $\alpha$ -dihydrotestosterone
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpm	disintegrations per minute
EDTA	ethylenediaminetetracetic acid
ER	oestrogen receptor
ER $\alpha$	oestrogen receptor alpha
ER $\beta$	oestrogen receptor beta
FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid
HPLC	high performance liquid chromatography
l	litre
M	molar
min	min
ml	millilitre
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
PBS	phosphate buffered saline
PCR	polymerase chain reaction



RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
s	second
T	testosterone
TBE	Tris-borate-EDTA
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminethane
Triton X-100	octyl phenoxy polyethoxyethanol
μl	microlitre

## **Chapter 1**

### **Introduction**

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- 1.1 Anatomy, embryology and functions of the prostate
- 1.2 Endocrinology of the prostate
- 1.3  $5\alpha$ -reductase
- 1.4 Aromatase
- 1.5  $17\beta$ -hydroxysteroid dehydrogenase
- 1.6 Steroid receptors
- 1.7 Benign prostatic hyperplasia
- 1.8 Management of benign prostatic hyperplasia
- 1.9 Experimental models of benign prostatic hyperplasia
- 1.10 Objectives of Thesis

## **1.1 Anatomy, Embryology and Functions of the Prostate**

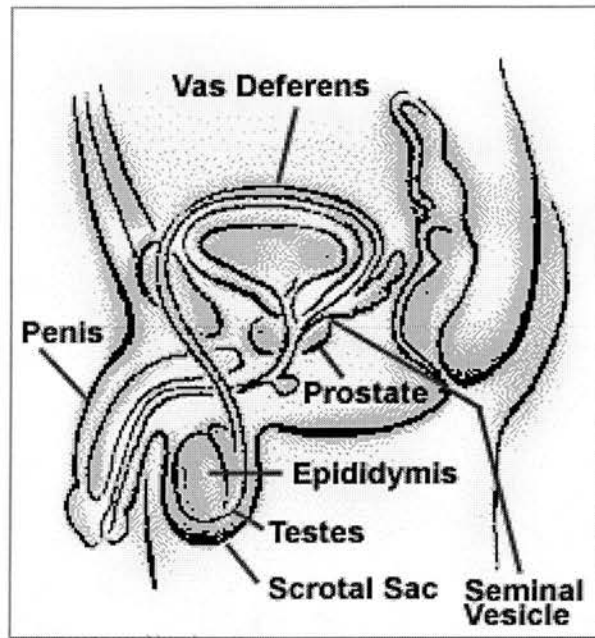
### **1.1.1 Gross anatomy**

Situated inferior to the urinary bladder and posterior to the symphysis pubis, the human prostate gland is approximately the size of a walnut. It is shaped like an inverted pyramid surrounding the uppermost (prostatic) part of the urethra. The gland has a fibromuscular capsule and is pierced by two ejaculatory ducts, through which the seminal vesicles expel their contents during ejaculation into the prostatic urethra (fig. 1.1).

### **1.1.2 Histology**

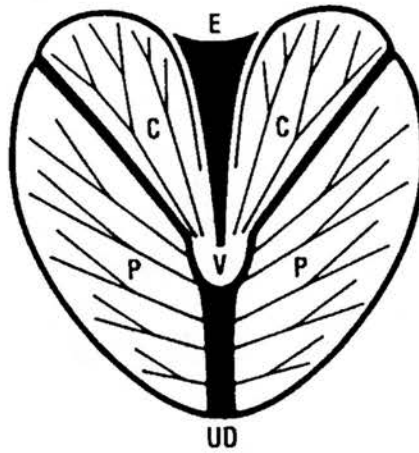
McNeal (1968) identified three distinct histological zones within the human prostate: peripheral zone, central zone, and transition zone (fig. 1.2). The peripheral zone in the normal gland comprises approximately 65% of the total prostatic volume and its histological appearance is characterised by small acini lined by columnar epithelial cells. The second largest component is the central zone, accounting for about 25% of normal prostatic volume. It surrounds the ejaculatory ducts and is characterised by large irregular acini lined by cuboidal epithelium. The smallest zone, transition zone, comprises only 5-10% of normal prostatic volume and is composed of two lobules found on either side of the prostatic urethra. Benign prostatic hyperplasia (BPH) is believed to arise from the transition zone whereas malignancy may affect any or all of the three zones with the majority of cases originating from the peripheral zone.

The prostate is an aggregate of 30 to 50 tubuloalveolar glands embedded in a fibromuscular stroma (fig. 1.3); these glands drain into the prostatic part of the urethra. Morphometric analysis showed that the normal adult prostate consists of 45-60% stromal tissue, 30-35% acinar lumen and 10-20% epithelial tissue (Uson et al. 1991).

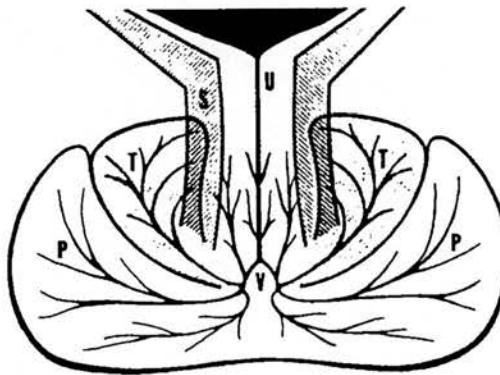


**Fig. 1.1** Diagram of the male reproductive system in sagittal section.

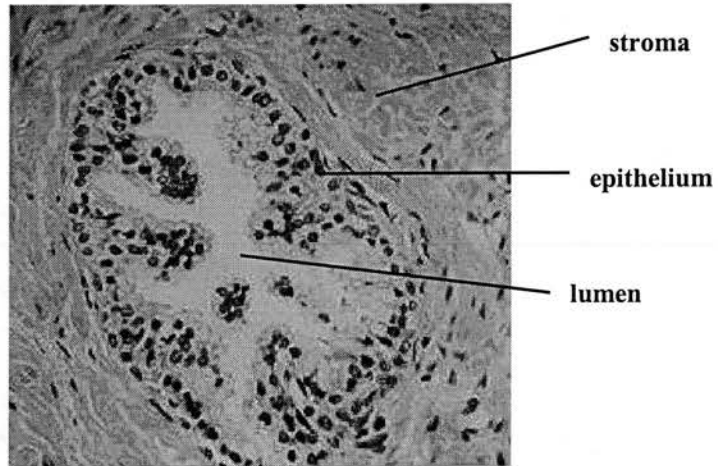
(a) coronal section



(b) oblique-coronal section



**Fig. 1.2 Schematic diagrams of (a) coronal and (b) oblique-coronal sections of the human prostate gland.** The verumontanum (V) is shown with its relationship to the ductular network draining the central zone (C), peripheral zone (P) and transition zone (T). E, ejaculatory duct; S, preprostatic sphincter. U, urethra; UD, distal urethra. Adapted from Uson et al. (1991)



**Fig. 1.3 Histology of normal human prostate.** The glandular lumen is lined by epithelial cells, which are embedded in a stroma consisting mainly of fibroblasts and smooth muscle cells. Haematoxylin and eosin staining.

The prostatic epithelium consists of two cell types. The luminal cells are columnar or cuboidal secretory cells lining the lumen whereas the basal cells are either trigonal or flattened cells situated below or between luminal cells. It has been suggested that basal cells represent precursors of luminal cells (Robinson et al. 1998). The cells of the underlying stroma vary from almost exclusively fibroblasts to almost exclusively smooth muscle cells with a mixture of both cell types in most areas.

#### 1.1.3 Embryology and development

The human prostate begins its morphogenesis at about the twelfth week of development. All tissues within the prostatic capsule are traditionally thought to arise from the urogenital sinus. The only exception to this is the intraprostatic vasa deferentia and ejaculatory ducts, which are of Wolffian duct origin. However, it has been proposed that the central zone of the prostate may also be of Wolffian duct derivation (McNeal 1968). Embryonic development of the prostate is under the influence of testicular androgens (McNeal 1989).

Before puberty, the immature prostate is small in size and growth is minimal; distinct histological zones are not well differentiated in the pre-pubertal prostate. During puberty, the large amount of androgens secreted from the testes stimulates prostatic cells to undergo morpho-functional maturation, giving rise to the various histological zones and functional tubuloalveolar glands. The prostate reaches its full size and mature morphology at the age of 18 to 20 (Uson et al. 1991).

#### 1.1.4 Functions

More and more is known about the pathology of common diseases of the prostate. However, two simple questions remain unanswered - why do we have a prostate, and what does the gland do? The gland is not essential for life but is functionally associated with reproduction. It produces a thin alkaline fluid, which

contributes to the volume of seminal fluid. At the time of ejaculation, smooth muscle cells in the prostatic capsule and stroma contract, and the secretion from the many tubuloalveolar glands is expelled into the prostatic urethra. Prostatic secretion contains many substances including citric acid, prostaglandins, zinc and various enzymes including amylase, fibrinogenase, acid and alkaline phosphatases and is believed to neutralise the acidic environment of the vagina (Blandy & Lytton 1986). However, the precise functions of these substances are not fully understood.

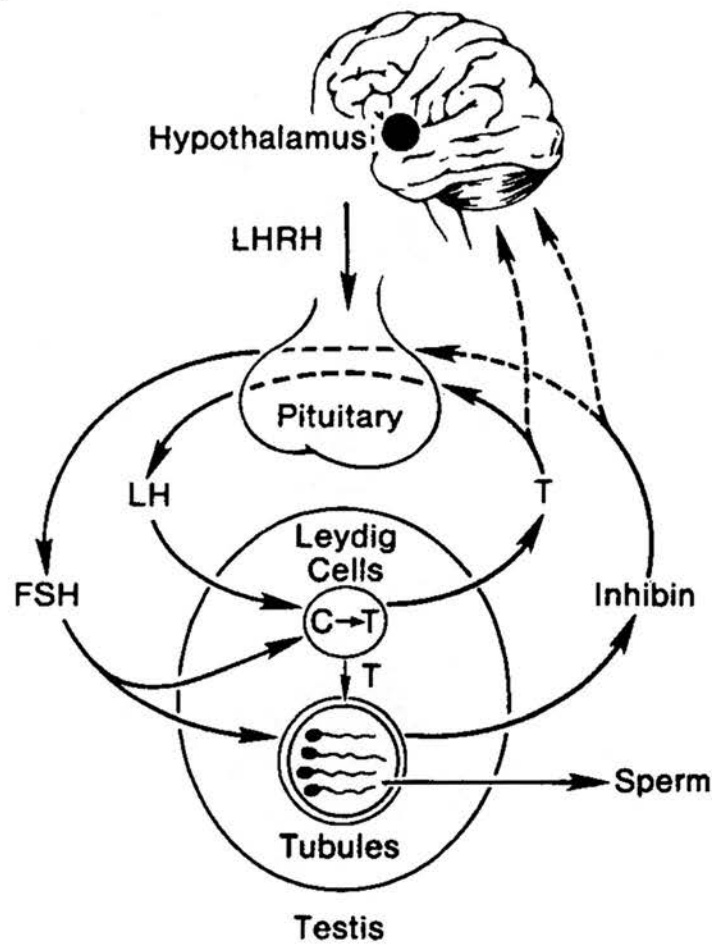
## **1.2 Endocrinology of the Prostate**

Although little is known about the physiological functions of the prostate, the maintenance of normal growth and development of this gland is widely accepted to be under the influence of testicular androgens. The essential role of androgens is illustrated by patients with the complete androgen insensitivity syndrome, also known as testicular feminisation. The main phenotypic characteristics of human males with testicular feminisation are female external genitalia, a short and blind-ending vagina, absence of Wolffian duct-derived structures and absence of a prostate (Brinkmann 2001). Moreover, in patients suffering from benign prostatic hyperplasia (BPH), prostate volume was decreased by the administration of the antiandrogen flutamide (Stone & Clejan 1991). Because androgens are crucial to the growth and development of the prostate, an account of their production and metabolism is outlined below.

### **1.2.1 Regulation of androgen secretion**

In the normal male, the major source of circulating androgens is the testes, with a very small contribution from the adrenal glands (Griffin & Wilson 1992). Testicular function is regulated by the two pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (fig. 1.4). LH stimulates Leydig cells to convert cholesterol to testosterone (section 1.2.2), which is then secreted into the bloodstream. Within the testis, testosterone acts synergistically with FSH on Sertoli cells to maintain spermatogenesis.





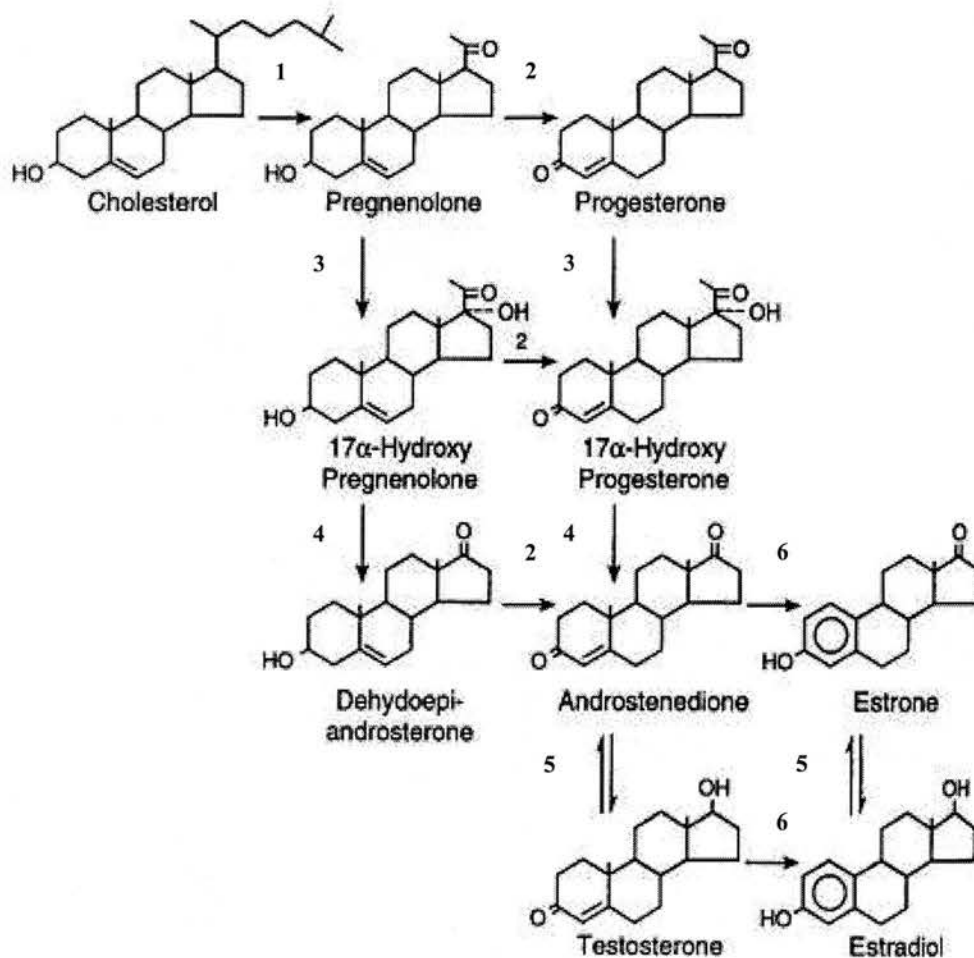
**Fig. 1.4 The human hypothalamic-pituitary-testicular axis.** The schematic diagram indicates the site of action of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Testosterone (T) is synthesised from cholesterol (C) in Leydig cells of the testis. Both testosterone and inhibin influence the pituitary gland and the hypothalamus by a negative feedback mechanism. LHRH, luteinizing hormone-releasing hormone. Adapted from Griffin & Wilson (1992).

Secretion of the two gonadotrophins is regulated by luteinizing hormone-releasing hormone (LHRH) secreted by the hypothalamus (fig. 1.4). The testis exerts an influence on the hypothalamic-pituitary unit by a negative feedback mechanism via both testosterone and inhibin, the latter being secreted by Sertoli cells of the testis. Other steroids, including dihydrotestosterone and oestradiol, can also affect this feedback control.

Another source of circulating androgens is the adrenal glands. The major adrenal androgens are dehydroepiandrosterone (DHEA), DHEA-sulphate and androstenedione. Although they are weak androgens compared with testosterone or dihydrotestosterone (Orth et al. 1992), these steroids are potential precursors of more active metabolites (fig. 1.5). Oesterling et al. (1986) demonstrated that the adrenal glands did not have a significant stimulatory effect on the human prostate and were not capable of maintaining prostatic growth without testicular androgens.

### 1.2.2 Androgen and oestrogen biosynthesis

The principal testicular androgen is testosterone. Besides testosterone, the testis also secretes smaller amount of other steroids including androstenedione, dihydrotestosterone, oestradiol and oestrone. In Leydig cells of the testis, testosterone is synthesised from cholesterol (fig. 1.5), which can either be locally produced from acetate or derived from the plasma pool of cholesterol. The first reaction in converting cholesterol to C-18, C-19 and C-21 steroids involves the cleavage of a 6-carbon group by cholesterol side-chain cleavage enzyme to form pregnenolone. Figure 1.5 shows that there are two possible pathways for the conversion of pregnenolone to androstenedione, one via the formation of dehydroepiandrosterone (DHEA) ( $\Delta 5$  pathway) and the other via the formation of progesterone ( $\Delta 4$  pathway). The predominant pathway in human testis is believed to be the  $\Delta 5$  pathway (Griffin & Wilson 1992). Androstenedione can then be reduced to the potent androgen testosterone by  $17\beta$ -hydroxysteroid dehydrogenase. Aromatase in the testis converts a small proportion of testosterone and androstenedione to oestradiol and oestrone respectively.



**Fig. 1.5 Pathways of androgen and oestrogen biosynthesis.** Numbered arrows represent enzymes involved in steroid conversion: (1) cholesterol side-chain cleavage enzyme; (2) 3 $\beta$ -hydroxysteroid dehydrogenase- $\Delta^{4,5}$ -isomerase; (3) 17 $\alpha$ -hydroxylase; (4) 17,20-desmolase; (5) 17 $\beta$ -hydroxysteroid dehydrogenase; (6) aromatase.

Of the circulating oestrogens in men, 75-90% arises from peripheral conversion of testosterone to oestradiol and androstenedione to oestrone in adipose, brain, bone and other tissues (Farnsworth 1999). The testes synthesise the remaining 10-25% of oestrogens by aromatisation of androgen precursors. The two natural oestrogens in man, oestradiol and oestrone, are interconverted by 17 $\beta$ -hydroxysteroid dehydrogenase (fig. 1.5).

The majority of circulating steroid hormones are bound to plasma proteins. The major binding proteins are cortisol-binding globulin (CBG), sex hormone-binding globulin (SHBG) and albumin (Orth et al. 1992, table 1.1). The formerly held notion that the active fraction of testosterone is identical to the unbound fraction in plasma has been refuted by the findings that dissociation of protein-bound hormone can occur within the capillary bed (Pardridge 1981). Moreover, SHBG, which binds to steroid hormones (testosterone, dihydrotestosterone and oestradiol in particular), has been suggested a vehicle for incorporating steroid hormones into the cytoplasm of prostatic cells (Farnsworth 1999), implying that the actual active proportion of circulating hormones delivered to target cells is larger than the free plasma fraction.

	% unbound	% bound to		
		CBG	albumin	SHBG
Dehydroepiandrosterone	4.1	<0.1	92.4	3.4
Androstenedione	7.9	1.4	88.0	2.8
Testosterone	2.2	3.6	49.9	44.3
Dihydrotestosterone	0.9	0.2	39.2	59.7
Oestradiol	2.3	<0.1	78.0	19.6
Oestrone	4.0	<0.1	88.6	7.4

Table 1.1 Transport of steroid hormones in plasma of men. Adapted from Orth et al. (1992). CBG, cortisol-binding globulin; SHBG, sex hormone-binding globulin.

### 1.2.3 Androgen and oestrogen metabolism in prostate

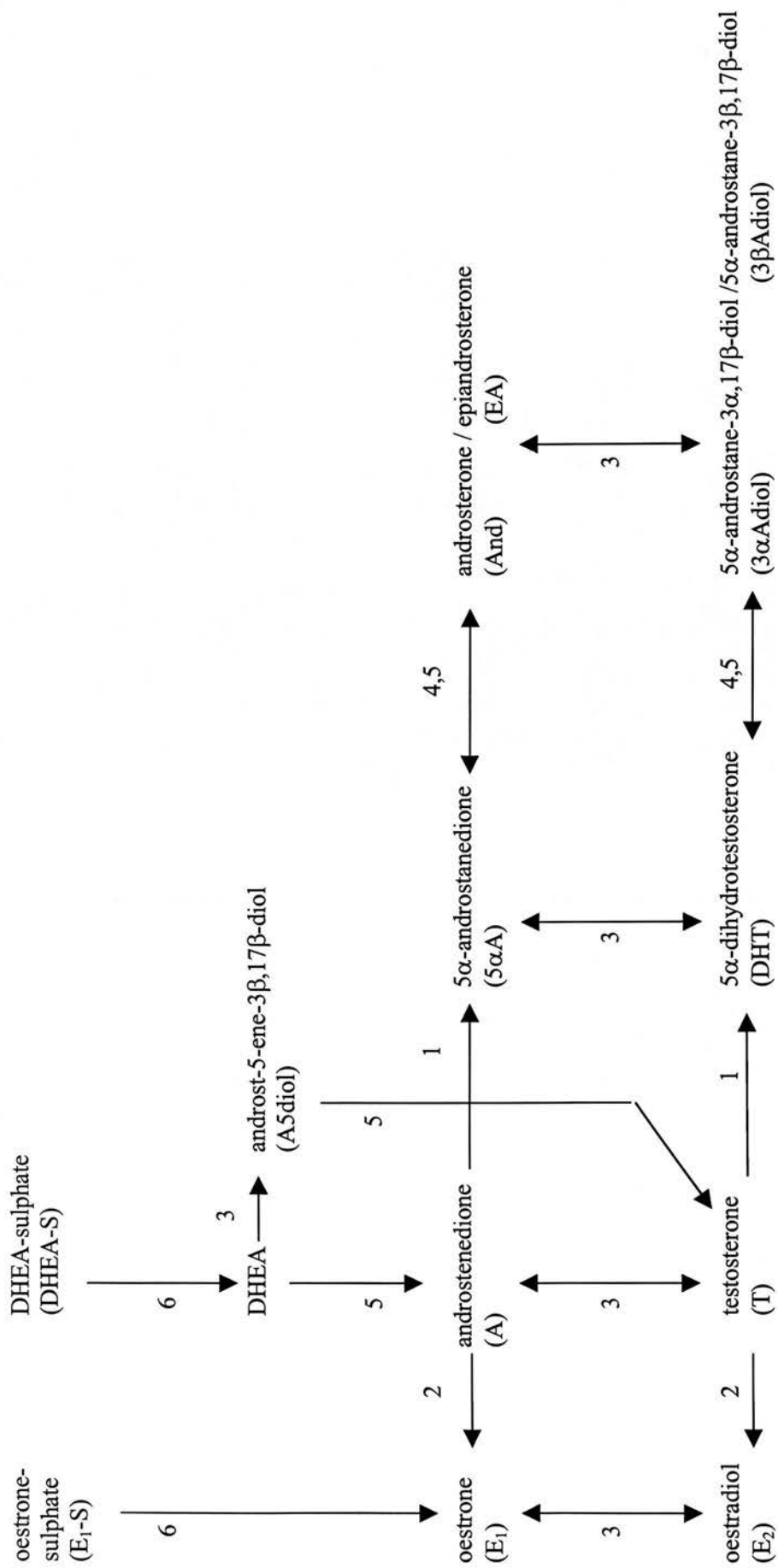
Steroid hormones, because of their hydrophobic nature, enter cells by diffusion. Before binding to specific intracellular receptors in the prostate, they may be subject to the action of steroid-metabolising enzymes. Indeed, these enzymes play important roles in steroid activation/inactivation or converting one class of steroids to another. A large number of androgen/oestrogen-metabolising enzymes have been reported in the prostate (Krieg et al. 1995, Stone et al. 1987); some potential pathways of steroid conversion are summarised in figure 1.6. Three of the enzymes which are of immediate relevance to the present thesis are discussed in the following sections.

### 1.3 $5\alpha$ -reductase

Steroid  $5\alpha$ -reductase, also known as 3-oxo- $5\alpha$ -steroid- $\Delta^4$ -oxidoreductase, is the major androgen metabolising enzyme in the human prostate (Krieg et al. 1995). It was reported to have higher steroid-metabolising activity than any of  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ HSD),  $3\alpha$ -hydroxysteroid dehydrogenase ( $3\alpha$ HSD),  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ HSD),  $6\alpha$ -hydroxylase and  $7\alpha$ -hydroxylase in prostate tissue.

#### 1.3.1 Two isozymes of $5\alpha$ -reductase

Two distinct isozymes of  $5\alpha$ -reductase, namely type 1 ( $5\alpha$ R1) and type 2 ( $5\alpha$ R2), have been identified in man (Andersson & Russell 1990, Andersson et al. 1991, Jenkins et al. 1992). Andersson & Russell (1990) first cloned the cDNA encoding  $5\alpha$ R1 (gene symbol *SRD5A1*). It is located on chromosome 5 and consists of 5 exons and 4 introns. The gene encodes a protein of 259 amino acids; over 40% of the amino acids are hydrophobic and only 60% are identical to those of the rat  $5\alpha$ R1 (Andersson & Russell 1990). A non-functional pseudogene for  $5\alpha$ R1 has been described and mapped to the X chromosome (Jenkins et al. 1991).



**Fig. 1.6 Potential pathways of steroid metabolism in prostate.** Numbers represent enzymes involved in steroid interconversion: (1) 5 $\alpha$ -reductase, (2) aromatase, (3) 17 $\beta$ -hydroxysteroid dehydrogenase, (4) 3 $\alpha$ -hydroxysteroid dehydrogenase, (5) 3 $\beta$ -hydroxysteroid dehydrogenase, (6) steroid sulphatase. DHEA, dehydroepiandrosterone.

The same group subsequently cloned the cDNA encoding 5 $\alpha$ R2 (gene symbol *SRD5A2*) from the prostate (Andersson et al. 1991). The human 5 $\alpha$ R2 gene also has 5 exons and 4 introns and encodes a hydrophobic protein of 254 amino acids, of which 50% are identical to those of human 5 $\alpha$ R1 and 77% identical to those of the rat type 2 isozyme (Andersson et al. 1991). Hydropathy plots of the two enzymes indicate no obvious transmembrane regions, suggesting that with hydrophobic amino acids distributed throughout their structures, these isozymes are intrinsic membrane proteins embedded in the lipid bilayer (reviewed in Russell & Wilson 1994).

Both isozymes convert testosterone to dihydrotestosterone (DHT) and androstenedione to 5 $\alpha$ -androstenedione (5 $\alpha$ A); a variety of other steroids with a 3-oxo- $\Delta^{4,5}$  structure including progestagens and glucocorticoids are also substrates of the two isozymes (Thigpen et al. 1993a, Levy et al. 1995). Levy et al. (1995) demonstrated that the substrate affinity order for human 5 $\alpha$ R1 (20 $\alpha$ -hydroxyprogesterone  $\geq$  progesterone  $>$  androstenedione  $\geq$  testosterone  $>$  corticosterone) was similar to that for human 5 $\alpha$ R2 (20 $\alpha$ -hydroxyprogesterone  $\geq$  progesterone  $>$  testosterone  $\geq$  androstenedione  $>$  corticosterone).

Different pH optima was one of the first characteristics that distinguished the two isozymes (Andersson et al. 1991). 5 $\alpha$ R1 exhibits a broader pH optimum (pH 7.0 - 8.5) than 5 $\alpha$ R2 (pH 5.0 - 5.5). The unusual acidic pH optimum of 5 $\alpha$ R2 *in vitro* is probably an experimental artefact due to disruption of membranes intimately associated with the isozyme (reviewed in Russell & Wilson 1994). Chinese hamster ovary (CHO) cells which had been transfected with a 5 $\alpha$ R2-encoding plasmid and gently permeabilised with digitonin showed similar 5 $\alpha$ -reductase activity at pH 5.0 and 7.0; in contrast, completely perforated cells had a typical acidic pH optimum at 5.0 (Thigpen et al. 1993a). The type 2 isozyme thus appears to function at a neutral pH within the cell and to shift to a form active at pH 5.0 upon cell lysis; this shift in pH requirement may reflect a conformational change in the isozyme. Nevertheless,



the neutral to basic and the acidic pH optima *in vitro* are in general diagnostic for the type 1 and type 2 isozymes respectively (Russell & Wilson 1994).

Between the two androgen substrates androstenedione and testosterone, 5 $\alpha$ R2 has similar  $K_m$  values for androstenedione and testosterone, whereas the type 1 isozyme has lower  $K_m$  value (and therefore higher affinity) for androstenedione than testosterone (table 1.2; Thigpen et al. 1993a, Levy et al. 1995). Also, 5 $\alpha$ R2 exhibits nearly identical catalytic efficiency ratios ( $V_{max}/K_m$ ) with the two androgen substrates, whereas  $V_{max}/K_m$  ratio for 5 $\alpha$ R1 is 6 to 11 times higher with androstenedione than testosterone (table 1.2). It is therefore likely that 5 $\alpha$ R1 also exhibits some selectivity towards androstenedione *in vivo*. Both isozymes utilize NADPH as a cofactor, with  $K_m$  values of 3 - 5  $\mu$ M for 5 $\alpha$ R1 and 7 - 10  $\mu$ M for 5 $\alpha$ R2 (Thigpen et al. 1993a).

study	substrate	5 $\alpha$ R1 (pH 7.0 or 7.5)			5 $\alpha$ R2 (pH 5.0)		
		$K_m$ ( $\mu$ M)	$V_{max}$ (nmol/min/ mg protein)	$V_{max}/K_m$ (nmol/min/ mg/ $\mu$ M)	$K_m$ ( $\mu$ M)	$V_{max}$ (nmol/min/ mg protein)	$V_{max}/K_m$ (nmol/min/ mg/ $\mu$ M)
Thigpen et al. (1993a)	A	0.3	5.7	19	0.2	2.4	12
	T	1.7	2.8	1.6	0.2	2.4	12
Levy et al. (1995)	A	1.0	0.40	0.40	0.8	0.09	0.11
	T	3.5-5.2	0.22	0.04-0.06	0.7	0.09	0.12

Table 1.2 Kinetic parameters of the two human 5 $\alpha$ -reductase isozymes. Kinetic constants were determined using cell homogenates prepared from CHO cells transfected with plasmids encoding either of the two 5 $\alpha$ -reductase isozymes. A, androstenedione; T, testosterone;  $K_m$ , Michaelis constant;  $V_{max}$ , maximal rate of reaction;  $V_{max}/K_m$ , catalytic efficiency.

### 1.3.2 $5\alpha$ -reductase and prostate development

Normal prenatal development of the prostate and Wolffian ducts requires androgen stimulation. While Wolffian duct differentiation is testosterone-dependent, dihydrotestosterone (DHT) is essential for the development of the prostate and male external genitalia. DHT is produced by  $5\alpha$ -reduction of testosterone and  $5\alpha$ R2 is the predominant isozyme detectable in fetal genital skin and male accessory sex glands including the prostate (Thigpen et al. 1993b). The importance of  $5\alpha$ R2 in normal prostate development is illustrated by mutations of the  $5\alpha$ R2 gene, which result in male pseudohermaphroditism. Affected individuals were shown to have male internal Wolffian-derived structures but female or ambiguous external genitalia and rudimentary prostates (Walsh et al. 1974, Andersson et al. 1991, Zhu et al. 1998). Transrectal ultrasonography and magnetic resonance imaging demonstrated that adult pseudohermaphrodites with  $5\alpha$ R2 deficiency had significantly smaller prostates than age-matched control males (reviewed in Zhu et al. 1998). Their prostates appeared as plate-like soft tissue structures on both imaging methods and transrectal prostate biopsies revealed fibrous connective tissue, smooth muscle and no epithelial tissue (Imperato-McGinley et al. 1992). No  $5\alpha$ R1 deficiency in human has yet been identified.

In contrast, male mice lacking  $5\alpha$ R2 or both  $5\alpha$ -reductase isozymes only had mild virilisation defects (Mahendroo et al. 2001). On the other hand, male mice homozygous for a targeted disruption of the  $5\alpha$ R1 gene were shown to have normal development of their reproductive organs including the prostate and normal endocrine profiles (Mahendroo et al. 1996). It was suggested that testosterone is the only androgen required for differentiation of the male urogenital tract in mice and that synthesis of DHT serves mainly as a signal amplification in this species (Mahendroo et al. 2001).

In comparison, data from female mice homozygous for a targeted disruption of the  $5\alpha$ R1 gene highlight the functions of the type 1 isozyme; in pregnant mice,

impairment of androgen  $5\alpha$ -reduction to non-aromatisable forms due to  $5\alpha$ R1 deficiency led to increased androgen aromatisation, elevation in plasma oestrogen levels and mid-gestational fetal loss (Mahendroo et al. 1997). This observation of increased plasma oestrogen concentrations in  $5\alpha$ R1-deficient mice indicates that a normal role for  $5\alpha$ R1 in the female mouse is to prevent the metabolism of placental androgens to oestrogens by converting aromatisable androgens to non-aromatisable forms. However, no specific role has yet been proposed for  $5\alpha$ R1 in the male or in humans.

### 1.3.3 Intraprostatic distribution of $5\alpha$ -reductase isozymes

Some studies have been carried out to examine if any differential spatial distribution of the two isozymes, which may suggest differential functions of the two isozymes, exists in the prostate. In situ mRNA hybridisation studies to localize mRNA distribution of both isozymes showed labelling predominantly in epithelial cells but also in the stromal compartment (Habib et al. 1998, Pelletier et al. 1998). Lehle et al. (1999) demonstrated both  $5\alpha$ R1 and  $5\alpha$ R2 mRNA in all of the peripheral, transition and central zones of normal prostate by RT-PCR. Most immunohistochemical studies have similarly localised both isozymes mainly in the epithelial layer; less immunoreactivity was detected in the stroma (Eicheler et al. 1994, Silver et al. 1994, Bonkhoff et al. 1996).

Using RT-PCR, Delos et al. (1998) detected mRNA of both  $5\alpha$ R1 and  $5\alpha$ R2 in primary cultures of epithelial cells grown from benign prostatic hyperplasia tissues; only  $5\alpha$ R1, but not  $5\alpha$ R2, mRNA was detectable with Northern blot analysis in the same study. Previous studies from the Prostate Research Group (University of Edinburgh) have shown that BPH-derived fibroblasts and epithelial cells in primary culture expressed mRNA detectable by RT-PCR and activity of the type 1 isozyme only (Bayne et al. 1998).

In summary, all three zones and both epithelial and stromal compartments of the human prostate have been reported to express both isozymes of  $5\alpha$ -reductase.

However, in interpreting these results, the limitations of each of the above techniques should be noted. For example, the presence of mRNA (detectable by either in situ hybridisation or Northern analysis) is not equivalent to the expression of protein. RT-PCR is a sensitive technique and can detect very small amount of mRNA present in tissue samples, whereas the usefulness of immunohistochemical studies is limited by specificity of the antibody used and antigen retrieval methods employed.

## **1.4 Aromatase**

### **1.4.1 Aromatase enzyme**

Aromatase, also known as oestrogen synthetase, catalyses the formation of aromatic C-18 oestrogens from C-19 androgen precursors. It is a microsomal member of the superfamily of enzymes collectively known as cytochrome P450 (Simpson et al. 1997). Associated with aromatase cytochrome P450 is the NADPH-cytochrome P450 reductase (reviewed in Simpson et al. 1994, Sasano & Harada 1998). The essentially ubiquitous NADPH-cytochrome P450 reductase transfers reducing equivalents from NADPH to any microsomal cytochrome P450 with which it comes into contact (Simpson et al. 1994). The aromatase reaction utilises 3 moles of oxygen molecules and 3 moles of NADPH for every mole of C-19 steroid metabolised, resulting in the aromatisation of A ring to give the phenolic structure characteristic of oestrogens (Simpson et al. 1997).

Both androstenedione and testosterone are substrates for aromatase. However, 5 $\alpha$ -reduced steroids such as 5 $\alpha$ -androstenedione and 5 $\alpha$ -dihydrotestosterone cannot serve as precursors because 5 $\alpha$ -reduction of the A ring precludes the completion of aromatisation. Kinetic studies have demonstrated that the enzyme has higher affinity for androstenedione than testosterone;  $K_m$  of aromatase in a variety of human tissues for androstenedione (25 – 100 nM) was slightly lower than  $K_m$  for testosterone (166 – 201 nM) (Schweikert 1979, Ackerman et al. 1981, Stone et al 1986, Krekels et al. 1991, Block et al. 1996).  $K_m$  for NADPH in human placental microsomes was reported to be 1  $\mu$ M (Reed & Ohno 1976).

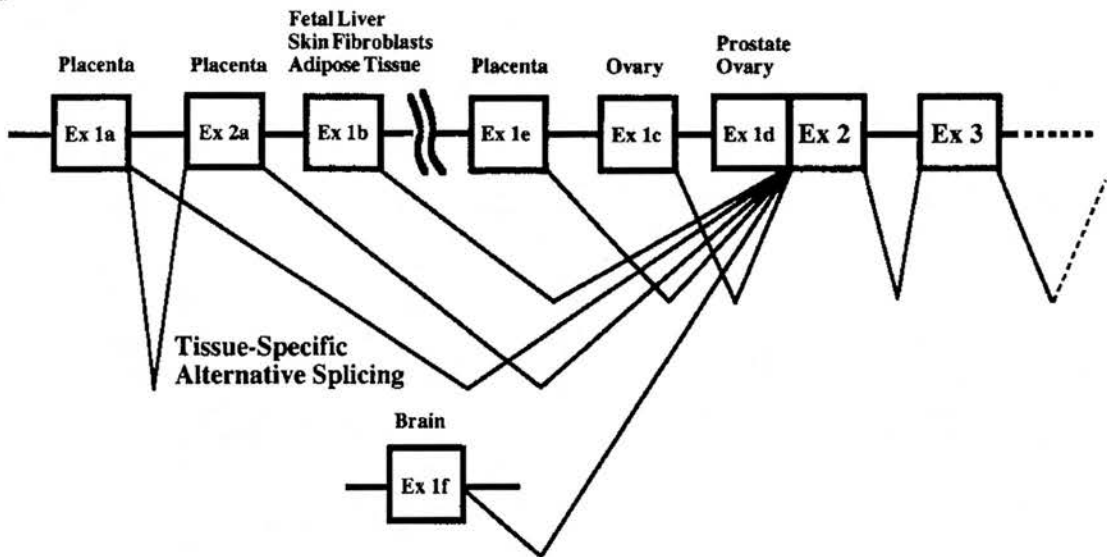
Aromatase is expressed in a wide variety of human tissues including adipose tissue, various regions of the brain, placenta, ovary and testis. In men, oestrogens are thought to be formed primarily by aromatisation in adipose tissue (section 1.2.2).

### 1.4.2 Aromatase (*CYP19*) gene

Within the cytochrome P450 superfamily of enzymes, aromatase is presently the sole member of the gene family 19, designated *CYP19*. This designation is based on the fact that the C-19 methyl group of androgens is the site of attack by oxygen. Corbin et al. (1988) cloned the full-length cDNA for *CYP19* from human placenta; the cDNA encodes a predicted protein of 503 amino acids. *CYP19* has been localised to human chromosome 15; it spans more than 120 kb of genomic DNA and comprises 10 exons (Sebastian & Bulun 2001). The coding region spans nine exons beginning with exon 2. Tissue-specific expression of aromatase is determined in part by alternative use of tissue-specific promoters, which give rise to mRNA with unique 5' noncoding ends (fig. 1.7; Simpson et al. 1997, Harada 1999). Numerous untranslated exons 1 have been identified. Two different systems of nomenclature of major splicing variants are currently in use (table 1.3); Harada's nomenclature is used in the following discussion. The predominant forms of various alternative exons 1 in tissues have recently been summarised by (Harada 1999) and Sebastian & Bulun (2001).

nomenclature		human tissues where splice variant is predominantly detected
Harada	Simpson	
1a	I.1	placenta
1b	I.4	fetal liver, adipose stroma, skin fibroblasts, bone
1c	I.3	ovary
1d	PII	ovary, testis, prostate
1e	I.2	placenta
1f	-	brain

Table 1.3 Two different nomenclatures of major aromatase splice variants. Adapted from Sasano & Harada (1998).



**Fig. 1.7 Structure of the human aromatase gene (*CYP19*) upstream of exon 3 showing alternative splicing patterns.** Ex, exon. Seven untranslated exons (Ex 1a – Ex 1f and Ex 2a) and the first two coding exons (Ex 2 and Ex 3) are shown. The most common sites of tissue-specific utilisation of alternative exon 1 are indicated. Adapted from Harada (1999). Exon 1f was later localised between exons 1b and 1e, approximately 30 kb upstream of exon 1e (Sebastian & Bulun 2001).

### 1.4.3 Aromatase expression in the prostate

Aromatase expression in the human prostate is controversial. Some have reported aromatase mRNA (Harada et al. 1993, Tsugaya et al. 1996, Hiramatsu et al. 1997) and activity (Schweikert 1979, Stone et al. 1986) in the prostate but others could not detect any enzyme activity in prostate tissues (Bartsch et al. 1987, Brodie et al. 1989).

The expression of aromatase mRNA has been reported in normal prostate, BPH and prostate cancer specimens (Harada et al. 1993, Tsugaya et al. 1996, Hiramatsu et al. 1997). A tissue-specific utilisation of exon 1d in the normal prostate was described by Harada et al. (1993). However, in another study, exons 1b, 1c and 1d (in decreasing order of prevalence) were reported in mRNA prepared from both BPH and cancer tissue samples (Hiramatsu et al. 1997). To date, it is not entirely clear which splice variant is the predominant one in human prostate.

To study the intraprostatic distribution of aromatase, two immunohistochemical studies using polyclonal antibodies have localised the enzyme mainly to the prostatic stroma (Matkin & Soloway 1992, Hiramatsu et al. 1997). Both normal and BPH tissues showed pronounced immunopositivity in the stroma but the epithelium was also stained when a higher antibody concentration was used (Matkin & Soloway 1992). Immunoreactivity was likewise observed by Hiramatsu et al. (1997) in stromal cells with marked heterogeneity among various regions of individual specimens; no immunoreactivity was detected in epithelial cells.

Although three studies (Smith et al. 1982, Bartsch et al. 1987, Brodie et al. 1989) have failed to detect any aromatase activity in BPH or prostate cancer tissues, aromatisation in normal, BPH and cancerous specimens have been shown by other investigators (Schweikert 1979, Kaburagi et al. 1987, Stone et al. 1986 & 1987, Hiramatsu et al. 1997). Stone et al. (1987) revealed higher aromatase activity in BPH than in cancer tissues, whereas Hiramatsu et al. (1997) reported no difference in aromatase activity between the two types of pathological tissues. Aromatase activity



was also detected in primary cultures of prostatic fibroblasts grown from BPH and prostate cancer tissues (Schweikert 1979).

#### 1.4.4 Abnormal aromatase expression

One way to understand the functional role of an enzyme is to look at individuals deficient in that particular enzyme. There are two well-documented cases of aromatase deficiency in adult males in the literature to date (Morishima et al. 1995, Carani et al. 1997). Both of the patients had decreased plasma levels of oestrogens, and either normal or increased levels of androgens including androstenedione, testosterone and dihydrotestosterone (table 1.4); the plasma levels of FSH, LH and androgens decreased after oestrogen supplement treatment (Carani et al. 1997).

hormone	plasma hormone levels reported	
	Morishima et al. (1995)	Carani et al. (1997)
E <sub>2</sub>	↓	↓
E <sub>1</sub>	↓	?
A	↑	↔
T (total)	↑	↔
T (free)	↔	?
DHT	↑	?
FSH	↑	↑
LH	↑	↔
DHEA-S	↔	↔

Table 1.4 Pre-treatment plasma concentrations of hormones in two men with aromatase deficiency compared with normal males. E<sub>2</sub>, oestradiol; E<sub>1</sub>, oestrone; A, androstenedione; T, testosterone; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; DHEA-S, dehydroepiandrosterone-sulphate. ↑, increased; ↓, decreased; ↔, normal; ?, not reported.



These two reports show that aromatase deficiency can lead to increased plasma concentrations of androgens, secondary to elevated levels of gonadotrophins; the role of oestrogens in the normal feedback control of plasma androgen levels in the male was also confirmed. The prostate phenotype was, however, not mentioned in either of the two case reports.

To further understand the role of aromatase, animal models of abnormal aromatase expression have been studied. Hormonal profiles in aromatase knockout (ArKO) mice were similar to those found in aromatase-deficient human males (Fisher et al. 1998, McPherson et al. 2001). The size of the prostate in ArKO mice was increased, when compared with the wild-type prostate, with histological evidence of epithelial hyperplasia (McPherson et al. 2001). In contrast, transgenic mice over-expressing aromatase were reported to have elevated E<sub>2</sub> levels, with reduced testosterone and FSH levels; their prostates were small in size and poorly developed when compared with the wild-type prostate (Li et al. 2001). These mouse models confirmed the importance of aromatase and oestrogens in the normal development of the prostate in mice. Taken together, the above animal studies and natural human models of abnormal aromatase expression emphasise that in considering a role for aromatase in prostate development, the effects of oestrogens on plasma levels of androgens (crucial for prostate embryogenesis and growth, section 1.2) must be taken into account.

### **1.5 17 $\beta$ -Hydroxysteroid Dehydrogenase**

Both androgens and oestrogens have the highest affinity for their receptors in the 17 $\beta$ -hydroxyl steroid form. 17 $\beta$ -hydroxysteroid dehydrogenase/17-ketosteroid reductase, hereafter called 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD), is therefore considered important in regulating the biological activity of androgens and oestrogens.

At present, no less than 10 different mammalian 17 $\beta$ HSDs have been identified; at least six types of 17 $\beta$ HSD have been cloned in human (Peltoketo et al. 1999, Labrie et al. 2000, Nordling 2001). 17 $\beta$ HSDs belong to at least two distinct enzyme families: the short-chain alcohol dehydrogenase family and the aldo-keto reductase family (Penning 1997). The primary structures of the various 17 $\beta$ HSD enzymes are not very similar to one another, with amino acid identity among types 1 to 5 17 $\beta$ HSD ranging from 15% to 26% (Labrie et al. 1997). Because of the large number of 17 $\beta$ HSD enzymes present in human, only a synopsis will be given for types 1 to 5, which have been more characterised than the other types, in the following discussion. In intact cells, the activity catalysed by each type of 17 $\beta$ HSD is thought to be almost exclusively unidirectional (reviewed in Peltoketo et al. 1999, Labrie et al. 2000). Whereas types 1, 3, and 5 catalyse the reductive reaction, types 2 and 4 catalyse the oxidative reaction.

#### 1.5.1 17 $\beta$ HSD type 1

17 $\beta$ HSD type 1, abundantly expressed in ovarian granulosa cells, is essentially an enzyme of oestradiol synthesis. It catalyses the conversion of oestrone (E<sub>1</sub>) to oestradiol (E<sub>2</sub>) with 100-fold higher affinity for C-18 than for C-19 steroids (reviewed in Penning 1997, Labrie et al. 2000). Immunostaining of 17 $\beta$ HSD type 1 was detectable in very few epithelial cells and no stromal cells of normal prostate tissue (Pylkkanen et al. 1994, Elo et al. 1996).

#### 1.5.2 17 $\beta$ HSD type 2

17 $\beta$ HSD type 2 has less substrate specificity than type 1 17 $\beta$ HSD and catalyses the conversion of E<sub>2</sub> to E<sub>1</sub>, testosterone to androstenedione, DHT to 5 $\alpha$ -androstenedione (5 $\alpha$ A) and androst-5-ene-3 $\beta$ ,17 $\beta$ -diol (A5diol) to DHEA (Peltoketo et al. 1999, Labrie et al. 2000). It also possesses 20 $\alpha$ HSD (Wu et al. 1993) and 3 $\beta$ HSD (Suzuki et al. 2000) activities. It is widely distributed in many tissues

(Peltoketo 1999). Using Northern analysis, Elo et al. (1996) reported highly variable 17 $\beta$ HSD type 2 mRNA expression in prostatic tissue specimens.

#### 1.5.3 17 $\beta$ HSD type 3

Predominantly expressed in the testis, 17 $\beta$ HSD type 3 converts androstenedione to testosterone; it is also capable of reducing 5 $\alpha$ A to DHT and E<sub>1</sub> to E<sub>2</sub> (Peltoketo et al. 1999). Its importance in testosterone biosynthesis is exemplified by male pseudohermaphroditism due to deficiency in 17 $\beta$ HSD type 3 (Zhu et al. 1998). Affected 46,XY individuals have testes and Wolffian duct-derived structures but ambiguous external genitalia. Their plasma androstenedione, FSH and LH levels are elevated, with low plasma testosterone levels. Plasma levels of E<sub>2</sub> and E<sub>1</sub> are normal to increased (reviewed in Zhu et al. 1998). Using Northern analysis, Elo et al. (1996) failed to detect 17 $\beta$ HSD type 3 mRNA in BPH or prostatic cancer tissue.

#### 1.5.4 17 $\beta$ HSD type 4

17 $\beta$ HSD type 4 is expressed in many human tissues including the prostate (Delos et al. 1998) and oxidises both androgens and oestrogens. It is a multifunctional enzyme, with 17 $\beta$ HSD and 2-enoyl-acyl-CoA hydratase activity and capable of facilitating the transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes (Peltoketo et al. 1999).

#### 1.5.5 17 $\beta$ HSD type 5

Unlike the above 4 types of 17 $\beta$ HSD, which belong to the short-chain alcohol dehydrogenase superfamily of enzymes, 17 $\beta$ HSD type 5 is a member of the aldo-keto reductase family (Peltoketo et al. 1999, Labrie et al. 2000). In fact, it is highly homologous to other members of the aldo-keto reductase family, including 3 $\alpha$ HSD types 1 and 3 as well as 20 $\alpha$ -HSD. It converts androstenedione to testosterone and possesses 3 $\alpha$ -HSD activity to some extent (Peltoketo et al. 1999). Using both in situ

mRNA hybridisation and immunohistochemistry, El-Alfy et al. (1999) detected strong 17 $\beta$ HSD type 5 labelling in the stromal fibroblasts and basal epithelial cells of normal and hyperplastic prostate; luminal epithelial cells were more variably labelled.

In summary, 17 $\beta$ HSD types 2, 4 and 5 have been reported in the human prostate. Whereas type 1 17 $\beta$ HSD converts E<sub>1</sub> to E<sub>2</sub>, 17 $\beta$ HSD type 3 and type 5 can metabolise androstenedione to testosterone. Types 2 and 4 17 $\beta$ HSD, on the other hand, oxidise androgens and oestrogens with a 17 $\beta$ -hydroxyl group, including E<sub>2</sub>, testosterone and DHT (table 1.5; Labrie et al. 1997). Other types of 17 $\beta$ HSD are less well reported in the literature. Human 17 $\beta$ HSD type 7 cDNA has also been cloned and mRNA of 17 $\beta$ HSD type 7 was detectable using RT-PCR in human prostate tissue (Krazeisen et al. 1999).

type	predominant activity	putative function in steroid metabolism	expression in prostate
1	reductase	oestradiol production	present in a few epithelial cells only <sup>(a)</sup> (Pylkkanen et al. 1994, Elo et al. 1996)
2	dehydrogenase	inactivation of androgens and oestrogens	present but mRNA levels highly variable between specimens <sup>(b)</sup> (Elo et al. 1996)
3	reductase	testosterone production	not detectable <sup>(b)</sup> (Elo et al. 1996)
4	dehydrogenase	inactivation of androgens and oestrogens	present <sup>(b)</sup> (Delos et al. 1998)
5	reductase	testosterone production	strongly immunopositive in basal epithelial cells and stromal fibroblasts; staining more variable in luminal epithelial cells <sup>(a)</sup> (El-Alfy et al. 1999)

Table 1.5 Characteristics of 17 $\beta$ HSD types 1 to 5 and their expression in human prostate. Method of detection in prostate: <sup>(a)</sup>immunohistochemistry, <sup>(b)</sup>Northern analysis.

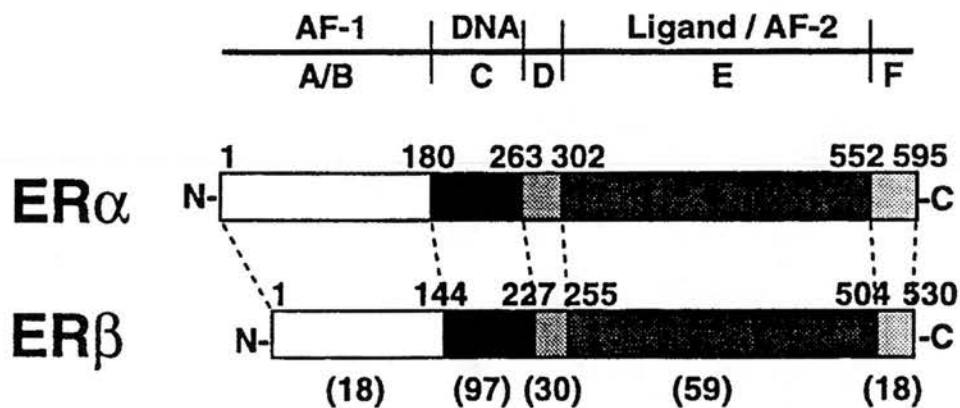
Two studies have investigated the expression of various types of 17 $\beta$ HSD in BPH-derived epithelial cells in primary culture (Delos et al. 1995, Delos et al. 1998). Types 2 and 4 17 $\beta$ HSD mRNA were present in epithelial cells in culture; neither type 1 nor type 3 was detectable by Northern analysis. Other types of 17 $\beta$ HSD were not examined in either of the two studies.

## **1.6 Steroid Receptors**

Steroid hormones, including androgens and oestrogens, exert their actions directly on target gene expression by binding to specific intracellular receptors which function as hormone-inducible transcription factors. Steroid receptors belong to the steroid/nuclear receptor (NR) superfamily, which includes the class I NR, the steroid receptors, e.g. androgen, oestrogen, glucocorticoid, mineralocorticoid and progesterone receptors, and the class II NR, e.g. retinoic acid receptor, retinoid X receptor, vitamin D receptor, thyroid receptor, and peroxisome proliferator activated receptor (PPAR). The NR superfamily also includes numerous 'orphan receptors', denoted as such because their endogenous ligands are unknown (Evans 1988, reviewed in Klinge 2000). In the following discussion, oestrogen receptor (ER) and its mechanism of action will be used as an example for steroid receptors in general.

### **1.6.1 General structural characteristics**

Steroid receptors have evolutionarily conserved similarities in a series of structural domains. For example, oestrogen receptors (ER $\alpha$  and ER $\beta$ ) have six domains named A to F from the N- to the C-terminus (fig. 1.8 and section 1.6.3, Krust et al. 1986, Green & Chambon 1986). The three major functional domains of the ER are: 1) an N-terminus (domains A and B) that modulates transcription by its activation function-1 (AF-1); 2) a central DNA-binding domain (DBD, consisting of the C domain), whose zinc fingers interact directly with target DNA; and 3) the ligand-binding domain (LBD, domain E) that contains activation function-2 (AF-2) (reviewed in Klinge 2000, Katzenellenbogen et al. 2000).

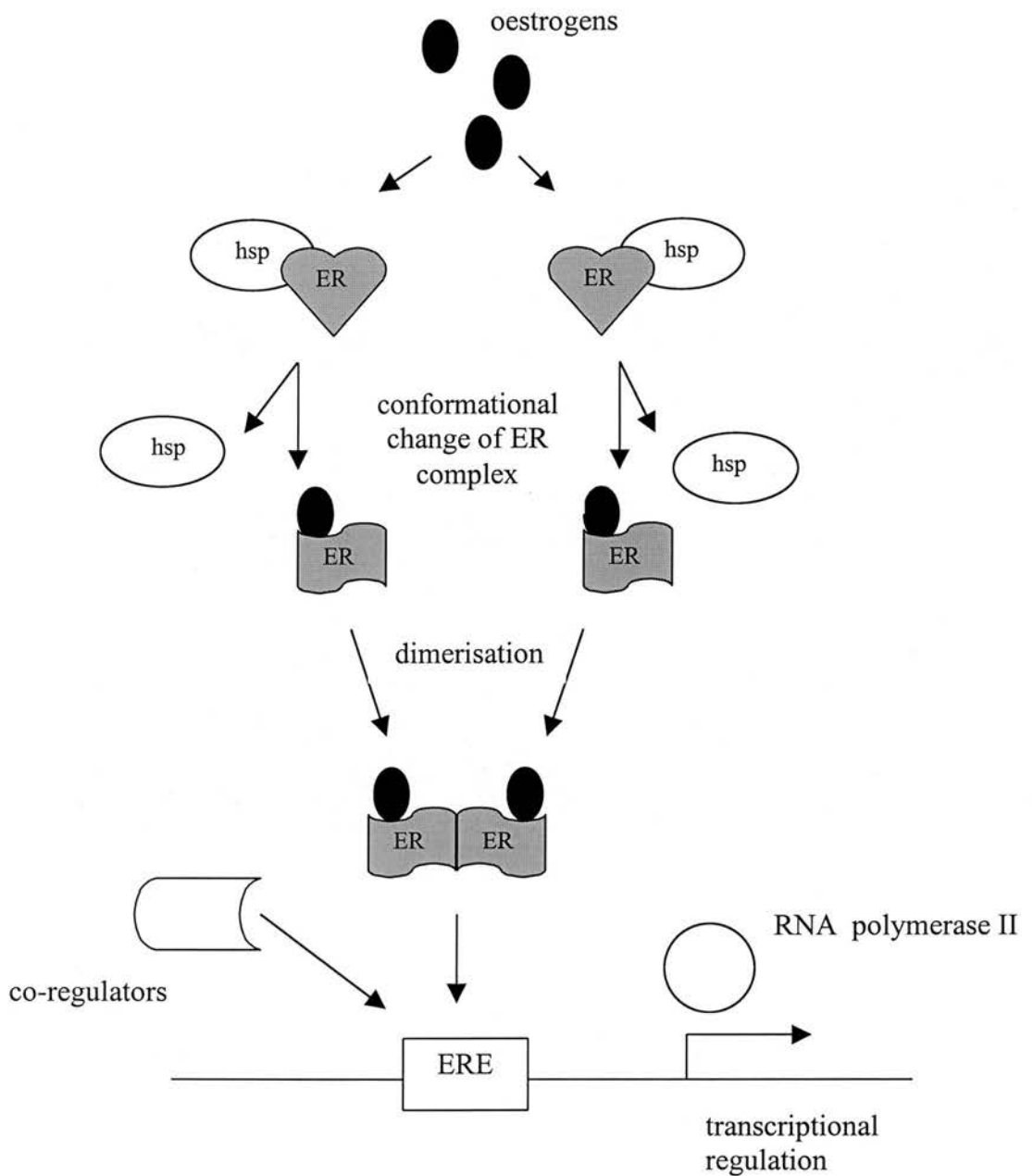


**Fig. 1.8 Schematic representation of the structural and functional domains of human oestrogen receptors (ER $\alpha$  and ER $\beta$ ).** Numbers of amino acids from N-terminus corresponding to the boundaries of various domains are indicated. The percentages of amino acid homology between structural domains A to F in the two ER subtypes are indicated by numbers in parentheses. AF, activation function. Adapted from Katzenellenbogen et al. (2000).

### 1.6.2 Mechanism of action

The unbound steroids are generally believed to diffuse through the cell membrane. It is currently not clear whether a transport protein is required for the movement of hydrophobic steroid molecules through the aqueous cytoplasm into the nucleus. ER resides in the nucleus in an unliganded state and is thought to be complexed with other proteins, e.g. hsp90 (reviewed in Katzenellenbogen et al. 2000). Binding of oestrogen to ER activates the receptor to undergo conformational changes to form an 'activated' ER, presumably by dissociation of hsp90 and other proteins (Williams & Franklyn 1994). Liganded ER $\alpha$  and ER $\beta$  can then form homodimers or heterodimers and bind to specific DNA sequences called oestrogen response elements (EREs) (fig. 1.9); the minimal consensus ERE sequence is a palindromic inverted repeat of 5'-GGTCAnnnTGACC-3', where n is any nucleotide (Katzenellenbogen et al. 2000). Examples of human genes whose promoters contain functional EREs include progesterone receptor, pS2 and oxytocin (Katzenellenbogen et al. 2000, Klinge 2000).

Initiation of transcription is a complex event occurring through the co-operative interaction of multiple factors at the target gene promoter (fig. 1.9). When bound to an ERE, ER interacts with transcription factors and other proteins, including co-activators, that may promote the assembly of the transcription initiation complex. Once the transcription initiation complex is complete, RNA polymerase II can initiate transcription (Klinge 2000). It has been suggested that oestrogen target cells express different levels of ERs, co-activators and co-repressors in order to achieve fine-tuning of target gene transcription in response to oestrogen stimulation (Klinge 2000).



**Fig. 1.9 Schematic model for oestrogen receptor (ER) action.** Oestrogens bind to ER, inducing the latter to undergo conformational changes and to dissociate from other proteins of the ER complex including heat-shock protein (hsp). Two liganded ER molecules then dimerise and bind to specific DNA sequences known as oestrogen response element (ERE). RNA polymerase and coregulatory proteins are then recruited to regulate gene transcription.



### 1.6.3 Two oestrogen receptor subtypes

In 1996, the cloning of a new member of the nuclear receptor superfamily from the rat prostate (Kuiper et al. 1996) and human testis (Mosselman et al. 1996) was reported. It has been named oestrogen receptor beta (ER $\beta$ ) to distinguish it from the oestrogen receptor (ER $\alpha$ ) cloned from the breast cancer cell line MCF-7 (Green et al. 1986). The identification of ER $\beta$  has prompted re-evaluation of the molecular basis for oestrogen action in health and diseases.

The ER $\alpha$  gene (*ESR1*), located on chromosome 6, encodes a protein of 595 amino acids, whereas the ER $\beta$  gene (*ESR2*), on chromosome 14, encodes a slightly shorter protein of 530 amino acids; both *ESR1* and *ESR2* consist of 8 exons (Enmark 1997, reviewed in Saunders 1998). The two ER subtypes contain the same arrangement of functional domains with the most conserved region being the DNA-binding domain (97% identity; fig. 1.8), consistent with the receptors binding to similar DNA response elements (Klinge 2000). Their ligand-binding domains are relatively well conserved (59% identity), which explains their similar affinities for various oestrogens and oestrogenic compounds (table 1.6). However, their A/B domains and activation function-1 (AF-1) share 18% homology only, suggesting that their transcriptional activation of different oestrogen-responsive genes may show distinctly different patterns (Katzenellenbogen & Korach 1997).

Both ER subtypes have similar relative binding affinities for a number of oestrogenic compounds ( $E_2 > E_1 > E_3 > A5\text{diol} > 3\beta\text{Adiol}$ ; table 1.6). However, ER $\beta$  has a lower affinity for  $E_2$  than ER $\alpha$  does;  $K_d$  values of the rat ER $\beta$  (0.4 - 0.6 nM) are higher than the rat ER $\alpha$  (0.1 nM) (Kuiper et al. 1996, Kuiper et al. 1997). Similarly,  $K_d$  for the mouse ER $\beta$  (0.5 nM) is also slightly higher than the mouse ER $\alpha$  (0.2 nM) with  $E_2$  as ligand (Tremblay et al. 1997).

compound	relative binding affinity		
	ER $\alpha$ (human)	ER $\beta$	
		(human)	(rat)
oestradiol (E <sub>2</sub> )	100	100	100
diethylstilboestrol	236-468	221	295
oestrone (E <sub>1</sub> )	60	NA	37
oestriol (E <sub>3</sub> )	14	NA	21
tamoxifen	4-7	3	6
androst-5-ene-3 $\beta$ ,17 $\beta$ -diol (A5diol)	1-6	7	17
5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ Adiol)	3	NA	7
5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ Adiol)	0.07	NA	0.3
5 $\alpha$ -dihydrotestosterone (DHT)	0.05	NA	0.17
dehydroepiandrosterone (DHEA)	0.04	NA	0.07
5 $\alpha$ -androstanedione (5 $\alpha$ A)	<0.01	NA	<0.01
testosterone	<0.01	<0.01	<0.01
androstenedione	<0.01	NA	<0.01

Table 1.6 Relative binding affinity of various compounds for the two ER subtypes. Relative binding affinity value for oestradiol was arbitrarily set at 100. Adapted from Kuiper et al. (1997) and Kuiper et al. (1998). The ligand binding domains of human and rat ER $\beta$  share 93.4% homology (Enmark et al. 1997). NA, data not available.

#### 1.6.4 ER expression in prostate

Using in situ hybridisation, Ehara et al. (1995) localised ER $\alpha$  mRNA to the stroma of BPH; epithelial cells were not labelled. Three immunohistochemical studies have also localised the ER $\alpha$  mainly to the stroma of normal prostate and BPH but immunostaining in the epithelial compartment was less intense and more variable (Ehara et al. 1995, Bonkhoff et al. 1999, Royuela et al. 2001).

Whereas expression of ER $\beta$  in the rat is highest in prostate and ovary (Kuiper et al. 1997), ER $\beta$  expression was relatively low in human prostate as detected by Northern analysis (Enmark et al. 1997). Using in situ hybridisation, Enmark et al. (1997) reported low ER $\beta$  mRNA expression in the epithelium of normal prostate

while the stroma was not labelled. Immunostaining of ER $\beta$  was positive in 13%, 30% and 80% of epithelial cells in normal prostate, BPH and cancer tissues respectively (Royuela et al. 2001); the same study reported no ER $\beta$  staining of stromal cells in normal or BPH tissues but 12% of cancer stromal cells were immunopositive. Another study also localised ER $\beta$  to the epithelium and a few stromal cells in normal prostate tissue by immunohistochemistry (Pasquali et al. 2001a).

To summarise, it is unclear whether ER $\alpha$  and ER $\beta$  are localised exclusively to either of the stromal or epithelial compartments. It appears that both in situ mRNA hybridisation and immunohistochemical studies have localised ER $\alpha$  mainly, but not exclusively, to the stroma and ER $\beta$  predominantly to the epithelium, at least in normal and BPH tissues.

## **1.7 Benign Prostatic Hyperplasia**

Three disease categories are common in the prostate; they are benign prostatic hyperplasia (BPH), prostatitis and carcinoma. BPH is the primary concern of this thesis.

### **1.7.1 Epidemiology**

BPH patients, on average, first present with urinary symptoms in their 60s with symptoms of lower urinary tract obstruction. The percentage of men with pathologically identifiable BPH at autopsy increases every year in men between 41 and 90 years of age; in general, about 40-50% of the male population have histological evidence of BPH at the age of 50 (Uson et al. 1991). Autopsy studies of BPH in different parts of the world showed that the age-specific prevalence of BPH was similar in many countries including Austria, Denmark, England, India, Japan and the United States (Isaacs & Coffey 1989). This is in contrast to the wide variation seen in the prevalence of clinically diagnosed BPH; one reason for the variation is the lack of a widely accepted case definition of BPH (Guess 1995).

### 1.7.2 Pathology

The majority of hyperplasia arises from the transition zone and periurethral region. Most clinically significant cases consist of enlargement of the transition zone; whereas periurethral hyperplasia seldom reaches clinically significant size, except as an occasional midline mass at the bladder neck protruding into the bladder lumen (McNeal 1990). The hyperplastic tissue usually comprises a mixture of fibroleiomyomatous nodules and glandular areas. It has been suggested that abnormal proliferation begins most likely in the stroma, while the proliferation of the glandular epithelium is secondarily induced (Hepap 1992).

### 1.7.3 Pathogenesis

Only two factors are generally considered essential for the development of BPH, ageing and the presence of a testis. At least two approaches to BPH aetiology that emphasise different possible mechanisms have evolved over the years.

#### 1.7.3a Theories based on hormonal mechanisms

The observation that the prostate does not develop in patients with testicular feminisation syndrome or  $5\alpha R2$  deficiency highlights the importance of androgens, DHT in particular, in the normal growth and development of this gland. Since the early report (Siiteri & Wilson 1970) that intraprostatic DHT concentrations were higher in BPH tissue than in normal tissue, DHT has been considered a factor in promoting the development of BPH. However, Walsh et al. (1983) demonstrated that DHT levels were not higher in BPH than in normal prostate tissue and argued that previously reported differences in prostatic DHT levels were artefacts due to differences in sample collection methods. Nevertheless, in patients with BPH, inhibition of  $5\alpha R2$  activity decreased both intraprostatic DHT concentrations and the prostate size (Rittmaster 1994). It is therefore possible that DHT plays a permissive rather than causative role in BPH pathogenesis.

It is noteworthy that BPH manifests clinically at a stage in life when both testosterone and DHT levels are declining. While plasma androgen levels decrease gradually, plasma oestrogen levels remain more or less constant, resulting in gradually increasing ratios of oestrogens to androgens with advancing age (table 1.7; Gray et al. 1991).

	trend in plasma levels with advancing age (39-70 yr) <sup>d</sup>	mean plasma levels in BPH patients (nM)	mean BPH tissue levels (nmol/kg wet tissue weight)
Androstenedione	↓ 1.3%/yr	5.5 <sup>b</sup>	30.0 <sup>b*</sup>
Testosterone	↓ 0.4%/yr	18.4 <sup>a</sup> ; 20 <sup>b</sup>	1.3 <sup>a</sup> ; 14.1 <sup>b*</sup>
Dihydrotestosterone	↔	2.6 <sup>a,b</sup>	25.6 <sup>a</sup> ; 45.5 <sup>b*</sup>
DHEA	↓ 3.1%/yr	15 <sup>c</sup>	69 <sup>c</sup>
DHEA-sulphate	↓ 2.2%/yr	NA	NA
3 $\alpha$ Adiol	↓ 0.8%/yr	0.84 <sup>a</sup>	1.43 <sup>a</sup>
3 $\beta$ Adiol	NA	1.4 <sup>a</sup>	7.1 <sup>c</sup>
A5diol	NA	2.2 <sup>c</sup>	9.9 <sup>c</sup>
Oestradiol	↔	0.147 <sup>a</sup> ; 0.064 <sup>c</sup>	0.229 <sup>a</sup> ; 0.095 <sup>c</sup>
Oestrone	↔	0.098 <sup>c</sup>	0.198 <sup>c</sup>

Table 1.7 Mean androgen and oestrogen levels in plasma and prostate of men. <sup>(a)</sup>Ghanadian & Puah (1981); <sup>(b)</sup>Habib et al. (1976); <sup>(c)</sup>Voigt & Bartsch (1986); <sup>(d)</sup>Gray et al. (1991). ↓, decreasing trend with age; ↔, no change with age; NA, data not available; \*, prostate hormone levels expressed as nmol/kg dry tissue weight. DHEA, dehydroepiandrosterone; 3 $\alpha$ Adiol, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol; 3 $\beta$ Adiol, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol; A5diol, androst-5-ene-3 $\beta$ ,17 $\beta$ -diol.

With advancing years, therefore, the ageing prostate is influenced by a gradually changing oestrogen/androgen balance. In the 1970s and 1980s, a lot of attention was focused on the role for oestrogens in BPH pathogenesis and management. Oestradiol was shown to exert a synergistic effect with androgens in inducing glandular prostatic hyperplasia in castrated dogs (Walsh & Wilson 1976, DeKlerk et al. 1979), whereas castrated dogs treated with oestradiol alone developed squamous epithelial metaplasia in their prostates (DeKlerk et al. 1979). It was thus hypothesised that oestrogens might also exert a synergistic effect with androgens in inducing prostatic hyperplasia in man (Walsh & Wilson 1976, DeKlerk et al. 1979). In another study, diethylstilboestrol (a synthetic oestrogen) also induced squamous metaplasia of the epithelium in prostatic tissue isolated from aborted human fetuses and then grown in athymic male mice (Sugimura et al. 1988). However, the role of oestrogens in the pathogenesis of BPH, a predominantly stromal disease, is not yet clear. With the discovery of a second oestrogen receptor (ER $\beta$ ) in 1996 (Kuiper et al. 1996, Mosselman et al. 1996), interests have once again focused on the role of oestrogens and the two ER subtypes in prostatic pathophysiology.

Recently, Weihua et al. (2001) demonstrated that in normal mice, but not in ER $\beta$  knockout mice, treatment with the C-19 steroid 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ Adiol) led to decreased androgen receptor protein expression in the prostate; they suggested that 3 $\beta$ Adiol could be an important ligand for ER $\beta$  in the prostate. Indeed, plasma levels of 3 $\beta$ Adiol are about 10-fold higher than plasma E<sub>2</sub> and E<sub>1</sub> levels, whereas in BPH tissue, concentrations of 3 $\beta$ Adiol are >30-fold higher than those of E<sub>2</sub> and E<sub>1</sub> (table 1.7). Another C-19 steroid is androst-5-ene-3 $\beta$ ,17 $\beta$ -diol (A5diol). Plasma and prostatic levels of A5diol were shown to be even higher than those of 3 $\beta$ Adiol (table 1.7); it also has relatively high binding affinity for ER $\beta$  (table 1.6, p.33). However, the function of this putative weak oestrogen in the prostate is unknown.



### 1.7.3b Stromal-epithelial interaction theory

During embryonic development, epithelial cells are directed by stromal signalling towards functional differentiation (Cunha et al. 1987). McNeal (1978) proposed the embryonic reawakening hypothesis and suggested that some stroma-derived humoral factor might induce glandular budding and branching, giving rise to new alveoli in the transition zone and eventually hyperplastic nodules. It has also been proposed that hyperplasia can be induced by abnormal levels of growth factors secreted from the epithelial or stromal compartment; these growth factors include the epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Habib 1994). Supporting this hypothesis are experiments on the transgenic mouse model, in which introduction of the int-2 proto-oncogene (a member of the FGF gene family) into the mouse genome resulted in enlargement of the animal's prostate gland (reviewed in Habib 1994).

Although the above theories of BPH pathogenesis appear to differ from one another in mechanism, it is possible that hormonal changes and epithelial-stromal interactions may synergistically influence the growth and function of various prostatic cell types, leading to hyperplasia of both epithelial and stromal components of the prostate. For example, locally produced steroid metabolites might act as cross-talk signals between the epithelium and stroma, and thus contribute to the development of BPH. Recently, Labrie et al. (2000) proposed that in basal cells of the prostatic epithelium, the inactive precursor DHEA is converted to androstenedione (by 3 $\beta$ HSD) and then testosterone (by 17 $\beta$ HSD); this locally produced testosterone is then transported into luminal cells and is further converted to the potent androgen DHT by 5 $\alpha$ -reductase. Whether this type of local steroid metabolism can lead to development of hyperplasia awaits further investigation. To date, the pathogenesis of BPH remains largely unresolved.

## 1.8 Management of BPH

Until about 20 years ago, the only options for patients with lower urinary tract symptoms (LUTS) due to BPH, which include both irritative symptoms (urgency, frequency, nocturia and urge incontinence) and obstructive symptoms (hesitancy, weak stream, straining, prolonged micturition and urinary retention), were either 'watchful waiting' or surgical treatment. However, with the advent of effective and safe medical therapies, the first-line treatment for BPH patients today is in most cases medical rather than surgical. Many different classes of drugs are currently available for the treatment of BPH. Because of limited space, only the three most commonly used are discussed below, followed by a brief outline of surgical options.

### 1.8.1 $\alpha$ -adrenoceptor blockers

The principal motor control of the prostate is via action on  $\alpha_1$ -adrenoceptors, which are localised predominantly within the stromal compartment of the prostate (Chapple 2000).  $\alpha$ -blockers relax the smooth muscle found in the prostatic capsule, stroma, urethra as well as bladder neck and can therefore relieve some BPH symptoms. The most widely prescribed  $\alpha_1$ -antagonists include prazosin, alfuzosin, doxazosin and terazosin. They can be used in prostates of any size and have a rapid onset of action (Djavan & Marberger 1999). Important side-effects in up to 10% of patients include tiredness, dizziness and headache due to  $\alpha$ -blockade in the brain and cardiovascular system (Djavan & Marberger 1999).

### 1.8.2 $5\alpha$ -reductase type 2 ( $5\alpha$ R2) inhibitors

Finasteride is the only  $5\alpha$ R2 inhibitor widely available for the treatment of BPH. It inhibits the conversion of testosterone to DHT, which plays an important role in maintaining prostatic growth (reviewed in section 1.3.2). Finasteride has been shown to effectively decrease the volume of the prostate, reduce BPH symptoms and



improve urinary flow rate measurements (Rittmaster 1994). Its main side-effects are reduced libido and impotence.

### 1.8.3 Phytotherapy

Phytotherapy, or the medicinal use of plant extracts, for treating LUTS and BPH is common in continental Europe and is increasing in the Western Hemisphere. There are currently more than 30 phytotherapeutic agents commercially available. In Italy, phytotherapeutic agents represent nearly half of all the medications dispensed for the treatment of LUTS, compared with 5% each for  $\alpha$ -adrenoceptor blockers and 5 $\alpha$ R2 inhibitors. In Germany and Austria, phytotherapy is the first-line treatment for mild-to-moderate LUTS. In the USA, phytotherapeutic agents for the treatment of LUTS are readily available as non-prescription dietary supplements (Wilt 2000). Plants from which common phytotherapeutic compounds were extracted for LUTS management include dwarf American palm (*Serenoa repens*), South African star grass (*Hypoxis rooperi*), rye-grass pollen (*Secale cereale*), African plum (*Pygeum africanum*) and stinging nettle (*Urtica dioica*).

#### 1.8.3.1 Permixon<sup>®</sup>

Of all the phytotherapeutic agents available for the management of LUTS and BPH, the most widely researched one is Permixon<sup>®</sup>. Permixon<sup>®</sup> is the lipido-sterolic extract of the fruit of *Serenoa repens*. The predominant constituents are free fatty acids (approximately 90%) including oleic acid, lauric acid, myristic acid and palmitic acid; there are also small quantities of aliphatic alcohols (C<sub>26</sub>, C<sub>28</sub> and C<sub>30</sub>) and phytosterols including  $\beta$ -sitosterol, campesterol and stigmasterol (Neuzil & Cousse 1993).

Clinical studies have shown that compared with finasteride (a 5 $\alpha$ R2 inhibitor), Permixon<sup>®</sup> produced similar improvements in both urological symptoms and urinary flow measures in patients with BPH and was associated with fewer adverse side effects (Carraro et al. 1996, Wilt et al. 1998).

Very limited data are available on the pharmacokinetic properties of Permixon<sup>®</sup>, probably because it is a complex mixture of various compounds. In healthy young male volunteers, after oral administration of a single dose of 320 mg Permixon<sup>®</sup> (usual recommended dose for BPH patients), mean peak plasma concentrations of the drug and elimination half-life were estimated to be 2.6 mg/l and 1.9 h respectively (Plosker & Brogden 1996).

The mechanism of action of Permixon<sup>®</sup> is not entirely clear. It has been shown to inhibit both isozymes of 5 $\alpha$ -reductase in a baculovirus-directed insect cell system expressing either of the two isozymes (Iehle et al. 1995) and in human BPH-derived cells in primary culture (Bayne et al. 1999). The conversion of testosterone to androstenedione in human prostatic cells in primary culture was also inhibited by Permixon<sup>®</sup> (Delos et al. 1995). In a clinical study, Permixon<sup>®</sup>-treated patients were shown to have decreased levels of nuclear oestrogen receptors but no difference in concentrations of cytosolic oestrogen receptors in their prostate tissue compared with the placebo-treated group (Di Silverio et al. 1992). Other reported effects of Permixon<sup>®</sup> include inhibition of DHT binding to androgen receptor (Carilla et al. 1984) and reduction of prostatic concentrations of epidermal growth factor (Di Silverio et al. 1998).

#### 1.8.4 Surgical management

Transurethral resection of the prostate (TURP) has been a gold-standard treatment for BPH for many years. It started to develop in the early 20<sup>th</sup> century and remains the procedure of choice for patients with severe LUTS and those whose symptoms do not improve with non-surgical treatment. It involves the removal of prostatic tissue in a slice-by-slice manner through a transurethral resectoscope. Other options of surgical treatment include bladder neck incision and open prostatectomy.

## 1.9 Experimental Models of BPH

### 1.9.1 Animal models

Spontaneous prostatic hyperplasia occurs commonly in only two species, man and dog (Walsh & Wilson 1976). While the human disease often has a multinodular pattern with predominantly stromal tissue, prostatic hyperplasia in the ageing dog is a diffuse epithelial process with little stromal involvement. Prostatic hyperplasia can also be induced in younger dogs by a combination of androgens and oestrogens, resulting in cystic glandular hyperplasia (Walsh & Wilson 1976, DeKlerk et al. 1979).

A transgenic mouse model which over-expressed the fibroblast growth factor family member, int-2, was reported to develop prostatic hyperplasia (Tutrone et al. 1993). The histology of the enlarged murine prostate resembled that of the dog; it was characterised by epithelial hyperplasia and cystic enlargement of the glandular lumen with no fibromuscular involvement (Tutrone et al. 1993), which is quite different from the usual histological features found in human BPH. The prostate of aromatase knockout mice also showed epithelial hyperplasia (McPherson et al. 2001). Because of the complications associated with animal experimentation and the differences in pathology between animal models and human BPH, other models to study BPH pathogenesis have been developed over the years.

### 1.9.2 Immortalised cell lines

Most immortalised cell lines are populations of aneuploid cells. The most commonly used human prostate cell lines are PC3, DU145 and LNCaP, all of which are derived from prostate cancer metastases and are epithelial in origin (Kaighn et al. 1979, Stone et al. 1978, Horoszewicz et al. 1983). The PC3 cell line was isolated from a bone metastasis and its characteristics are consistent with a poorly differentiated adenocarcinoma (Kaighn et al. 1979). The DU145 cell line derived

from a metastatic brain lesion in a patient who had undergone oestrogen therapy for advanced prostate cancer (Stone et al. 1978). The LNCaP cell line was established from needle biopsies taken from a supraclavicular lymph node containing prostate cancer metastatic cells (Horoszewicz et al. 1983); it expresses a mutated androgen receptor that binds to androgens, oestrogens and progestagens (Veldscholte et al. 1992). Because all of these three cell lines derived from prostate cancer metastases, they are not considered ideal models of benign hyperplastic cells.

Other prostate cell lines have also been developed, one example of which is the BPH-1 cell line. BPH-1 is an immortalised cell line derived from primary human epithelial cell cultures obtained by transurethral resection (Hayward et al. 1995). Although cell lines, in general, are relatively easy to maintain, long-term culturing of cells alters their phenotypes with increasing passage numbers, and brings into question the reliability of cell lines as representative models of the *in vivo* situation.

### 1.9.3 Primary cultures of BPH-derived cells

Short-term primary cell cultures derived from fresh BPH tissues should, in theory, not be too different from their tissue of origin in phenotypes. They represent the various cell populations normally present in the tissue of origin and yet provide the advantages normally associated with monolayer cell cultures. Methods to separate epithelial cells from fibroblasts using enzymatic digestion of prostate tissue chips obtained from BPH patients are well established in the Prostate Research Group Laboratory, University of Edinburgh. The cell cultures have been characterised by microscopy and immunohistochemistry employing a number of antibodies to identify epithelial cells, fibroblasts and smooth muscle cells (Tsugaya et al. 1996a). These immunohistochemical characteristics were also confirmed at the beginning of this project and corresponded well with the various cell types found in histological sections. These cells in primary culture provide *in vitro* models to study prostatic cells which are BPH in origin. However, primary prostatic cell cultures are by no means ideal experimental models of BPH. Their phenotypes are not entirely identical to cells *in vivo*; for example, 5 $\alpha$ -reductase type 2 isozyme is not expressed

in either epithelial cells or fibroblasts in primary culture (Bayne et al. 1998) but has been demonstrated in both compartments of hyperplastic prostate tissue (reviewed in section 1.3.3). Compared with immortalised cell lines, it is also relatively difficult to maintain viable and proliferating epithelial cells after sub-culturing. Although surgical resection of the prostate is a common treatment for BPH, the supply of prostate chips fluctuates and depends on many logistic factors. Bacterial and fungal contamination due to infected tissues is another problem associated with primary cultures.

### **1.10 Objectives of Thesis**

There are two main questions which this thesis set out to answer and they are:

1. What is the function of 5 $\alpha$ -reductase type 1 isozyme in the human prostate?
2. What is the role of oestrogens in BPH pathogenesis?

To date, two distinct isozymes of 5 $\alpha$ -reductase, 5 $\alpha$ R1 and 5 $\alpha$ R2, have been identified in man; both are expressed in the prostate. The role of 5 $\alpha$ R2 is well established. It is the predominant isozyme in the human prostate, where it converts testosterone to the more potent androgen dihydrotestosterone, crucial for maintaining growth and function (section 1.3.2). In contrast to 5 $\alpha$ R2, the function of 5 $\alpha$ R1 in the prostate is still unknown. Recent data from female 5 $\alpha$ R1 knockout mice indicated that a normal role for 5 $\alpha$ R1 in the female mouse is to prevent the metabolism of placental androgens to oestrogens by converting aromatisable androgens to non-aromatisable forms (Mahendroo et al. 1997). Here the hypothesis that 5 $\alpha$ R1 also plays a role in regulating aromatase activity in human prostatic cells was tested, first in a co-transfection model and then in primary cultures of prostatic cells. To this end, because aromatase expression in the human prostate is controversial, aromatase mRNA expression and activity in prostatic cells were also examined.

The aim of the second part of this study was to determine the effects of oestrogens on prostatic cells and whether these effects can contribute to the development of BPH. Classically, oestrogen action is mediated by oestrogen

receptor (ER); the expression of both ER subtypes in prostatic cells was thus investigated. This was followed by experiments to explore any effects of oestrogens on prostatic cell proliferation. As BPH is a result of increased cell proliferation or decreased cell death or both, results from such experiments would shed light on any possible role of oestrogens in BPH pathogenesis. Recently, 5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ Adiol) was suggested as an important ligand for ER $\beta$  in the prostate (Weihua 2001), its effects on prostatic cell proliferation were therefore examined and compared to those of oestradiol.

Many drugs are currently available for the treatment of BPH, one of which is the phytotherapeutic agent Permixon<sup>®</sup>. Although it has been shown to be clinically efficient in reducing the size of hyperplastic prostate (section 1.8.4), the mechanism of Permixon<sup>®</sup> is not yet clear. Part of this project was to look at yet unknown effects of Permixon<sup>®</sup> on 5 $\alpha$ R1 activity, oestrogen anabolism and catabolism in prostatic cells.

## **Chapter 2**

### **Materials and Methods**

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- 2.1 Materials
- 2.2 Cell culture
- 2.3 Analysis of 5 $\alpha$ -reductase activity in tissue homogenates
- 2.4 Preparation of expression plasmids
- 2.5 Transfection studies
- 2.6 Steroid metabolism in prostatic cells in primary culture
- 2.7 Analysis of steroid metabolites
- 2.8 Isolation of ribonucleic acids from cell cultures
- 2.9 Analysis of nucleic acids
- 2.10 Preparation of Permixon<sup>®</sup>
- 2.11 Statistical analysis



## 2.1 Materials

Unless otherwise stated, all chemicals and reagents were purchased from Sigma (Poole, Dorset, UK)

### 2.1.1 Cell culture

#### 2.1.1a Cell lines

The PC3 cell line was isolated from a poorly differentiated bone metastasis of human prostatic carcinoma (Kaighn et al. 1979). The COS-1 cell line was derived from the simian kidney CV-1 cell line (Gluzman 1981). The CHO cell line was derived from a biopsy of an adult Chinese hamster ovary (Puck 1958). The C6 cell line was cloned from a rat glial cell tumor (Benda et al. 1968). All cell lines were obtained from the European Collection of Cell Cultures (ECACC), Centre for Applied Microbiology and Research, Salisbury, Wiltshire, UK and were regularly screened for mycoplasma contamination using a mycoplasma detection kit (Boehringer Mannheim UK, Lewes, UK).

#### 2.1.1b General consumables

All plasticware including tissue culture flasks, Universal containers, Erlenmeyer flasks and disposable pipettes was obtained from Bibby Sterilin (Staffordshire, UK). All media and solutions were sterile-filtered before use with either 0.2  $\mu\text{m}$  bottle-top filter or 0.2  $\mu\text{m}$  Acrodiscs supplied by Sigma and Gelman Sciences (Northampton, UK) respectively. Dulbecco 'A' phosphate buffered saline (PBS) was purchased from Unipath (Basingstoke, Hampshire, UK). 10x Trypsin-EDTA (0.5% w/v trypsin, 0.2% w/v EDTA and 0.85% w/v NaCl) was from Life Technologies (Paisley, Scotland, UK).



### 2.1.1c Cell culture media and supplements

RPMI 1640 medium, DMEM medium and WAJC 404 medium were obtained from Life Technologies. All media, unless specified otherwise, were supplemented with foetal calf serum (FCS), L-glutamine (200 mM), penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml), which were acquired from Life Technologies. Collagenase was obtained from Worthington Biochemical Corporation (Freehold, New Jersey, USA). MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was purchased from Promega (Southampton, UK).

### 2.1.2 Molecular biology

All materials utilised in reverse transcription (RT) and polymerase chain reaction (PCR) were from Promega (Southampton, UK). Restriction enzymes were also from Promega. PfuTurbo DNA polymerase was purchased from Stratagene (Amsterdam, the Netherlands). Agarose NA was obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Sterile screw cap Eppendorf tubes were purchased from Sarstedt (Beaumont Leys, Leicester, UK). All primers were synthesised by Oswel DNA Service (University of Southampton, Southampton, UK).

### 2.1.3 Steroids

All non-radiolabelled steroids were from Sigma. Radiolabelled steroids including [4-<sup>14</sup>C]-androstenedione, [4-<sup>14</sup>C]-dihydrotestosterone, [4-<sup>14</sup>C]-testosterone (all of S.A. 53.6 mCi/mmol) and [4-<sup>14</sup>C]-oestrone (S.A. 50.3 mCi/mmol) were obtained from NEN Life Science Products (Hounslow, UK). [4-<sup>14</sup>C]-5α-androstenedione was synthesised from [4-<sup>14</sup>C]-androstenedione as described in section 2.3.3. Tritium-labelled steroids, including [1,2,6,7-<sup>3</sup>H]-androstenedione (S.A. 100 Ci/mmol), [1,2,6,7-<sup>3</sup>H]-testosterone (S.A. 95 Ci/mmol), [2,4,6,7-<sup>3</sup>H]-oestrone (S.A. 101 Ci/mmol) and [2,4,6,7-<sup>3</sup>H]-oestradiol (S.A. 99 Ci/mmol) were from

Amersham (Little Chalfont, Bucks, UK). Radiolabelled steroids were purified by thin layer chromatography before use as described in section 2.3.3.

#### 2.1.4 Chromatography

##### 2.1.4a Thin layer chromatography (TLC)

ITLC™ SA polysilica acid gel impregnated glass fibre TLC plates were purchased from Gelman Sciences (Ann Arbor, Michigan, USA). Ultima Gold Liquid Scintillation Cocktail was supplied by Packard Bioscience (Pangbourne, Berks, UK). Diethyl ether, dichloromethane, ethanol and benzene were obtained from Fisher Scientific (Leicestershire, UK).

##### 2.1.4b High performance liquid chromatography (HPLC)

All HPLC grade solvents were supplied by Rathburn Chemicals (Walkerburn, Peebleshire, UK). Quicksafe Flow-2® scintillant was purchased from Zinsser Analytic (Maidenhead, Berks, UK)

## 2.2 Cell Culture

### 2.2.1 Cell lines

#### 2.2.1a Culture of cell lines

The COS-1, CHO, C6 and PC3 cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). The cell lines were grown in monolayer culture in 75 cm<sup>2</sup> tissue culture flasks in a humidified incubator (T305GF Assab; Kebo Assab AB, Solna, Sweden) maintaining

an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. All tissue culture procedures were carried out in a laminar flow cabinet (Howarth Air Engineering Ltd., Farnworth, Bolton, UK) using aseptic techniques and sterile, disposable plastic pipettes and pipette tips. To ensure that a sterile environment was maintained, the laminar flow cabinet was thoroughly cleaned before, during and after operations with a solution of 70% ethanol. In addition, all handling of cell lines was performed using disposable latex gloves.

Continuous cell growth was maintained through regular sub-culture 2-3 times per week. Upon achieving approximately 80% confluence, cells were harvested through trypsinisation, diluted 1:3 in medium and delivered into 3 fresh flasks. The sub-culture process involved the aspiration of spent medium from cells followed by 2 washes with phosphate buffered saline (PBS) and a 3 min incubation at 37°C in 2 ml 1x trypsin-EDTA (0.2 ml 10x trypsin-EDTA and 1.8 ml PBS). The cells were then detached through gentle agitation and 10 ml of the appropriate medium was added to the cell suspension. Following centrifugation at 2000 rpm for 5 min, resuspension of the pellet in 12 ml medium with repeated pipetting ensured cell suspension. 4 ml of this suspension was delivered to each flask, to which an additional 11 ml medium was added. The medium was changed every 48-72 h.

#### 2.2.1b Long term storage of cells

Cell lines could be stored for long periods by freezing in a gas phase liquid nitrogen storage container. Cells were prepared for freezing by trypsinisation as described above. The cells were pelleted by centrifugation at 2000 rpm for 10 min and the pellet was then resuspended in 1 ml freezing medium, which consisted of 10% dimethyl sulphoxide (DMSO) in FCS. The cells were mixed with the freezing medium by titration before being transferred to cryogenic ampoules (Corning) and stored at -70°C overnight. The cells were then transferred to the gas phase of liquid nitrogen.

### 2.2.1c Thawing of frozen cells

Frozen cells were thawed in a 37°C water bath. DMSO in the freezing medium was washed out of the thawed cell suspension by transferring it to a Universal containing 20 ml culture medium. The cell suspension was thoroughly mixed by vortexing prior to centrifugation at 2000 rpm for 10 min. The cell pellet was then resuspended in 10 ml culture medium and transferred to a 75 cm<sup>2</sup> cell culture flask.

### 2.2.2 Culture of epithelial cells and fibroblasts from human prostatic tissue

The primary culture of epithelial cells from prostatic acini in serum-free medium was first described by Chaproniere & McKeehan (1986). The protocol described here is based on the technique developed by Chaproniere & McKeehan (1986) and described by Habib et al. (1992). Verification of the purity of epithelial cells and fibroblasts was established by immunohistochemistry using a variety of mouse monoclonal anti-human antibodies as described by Tsugaya et al. (1996).

Prostate tissue was obtained with informed consent from patients undergoing transurethral resection of the prostate (TURP) for the treatment and relief of the symptoms of BPH. The prostate chips were immediately placed in 15 ml Transport Medium (RPMI 1640 medium supplemented with 5% FCS) and were stored for up to 7 days at 4°C before use. Pathology of prostate tissue was determined by routine examination of representative samples of excised tissue by histopathologists at the Department of Pathology, Western General Hospital, Edinburgh.

#### 2.2.2a Enzymatic dissociation of fibroblasts and epithelial cells

The prostate chips were washed twice in 25 ml PBS, weighed and cut into approximately 2 mm<sup>3</sup> cubes using sterilised scissors and forceps. The tissue was subsequently transferred to a sterile Erlenmeyer flask, washed with 20 ml Transport

Medium and finally suspended in 5 ml Transport Medium per gram of tissue. 2.5 ml filter-sterilised collagenase solution (600 units/ml) in Transport Medium was mixed with each gram of tissue and the flask placed in a 37°C shaking incubator (Luckham R300; Luckham Ltd., Burgess Hill, UK). The tissue was incubated for 20 h with gentle agitation to maintain the integrity of the acini. The digest was then triturated by repeated pipetting, decanted into sterile Universal containers and centrifuged at 2000 rpm for 10 min. The supernatant was decanted and the cell pellet resuspended in 20 ml PBS to wash out the collagenase. The cells were again centrifuged at 2000 rpm for 10 min and washed in PBS. These steps of centrifugation and washing were repeated prior to suspension of the pellet in 10 ml Transport Medium. The acini, containing epithelial cells, were sedimented by centrifugation at 800 rpm for 20 s. The supernatant contained aggregates of fibroblasts. The acini were carefully aspirated using a sterile Pasteur pipette and transferred to a fresh Universal container. The acini were spun down twice more and collected in the same manner.

#### 2.2.2b Epithelial cell culture

Epithelial cells were maintained in epithelial cell growth medium (EGM), each litre of which consisted of the following in distilled water:

11.04 g WAJC 404 medium powder

6.7 g HEPES

1.2 g sodium hydrogen carbonate

0.5 mg zinc stabilised insulin

20 µg cholera toxin

392 µg dexamethasone

10 µg epidermal growth factor

0.5% FCS (v/v)

100,000 units penicillin

100 mg streptomycin

pH of the growth medium was adjusted to 7.6 using 1M NaOH.

After enzyme dissociation, acinar deposits were resuspended in 5 ml EGM per gram of original tissue and plated out in 75 cm<sup>2</sup> tissue culture flasks. One flask was used for every 2-4 g tissue. The cultures were incubated at 37°C for 72 h before being supplemented with 10 ml EGM. After a further 48 h incubation, the spent medium could be replaced with fresh medium. Upon reaching 80% confluence, epithelial cells were harvested by trypsinisation using 1x trypsin-EDTA solution. Following the detachment of epithelial cells from the flask, the trypsin solution was inactivated using RPMI 1640 medium supplemented with 10% FCS. The cells were then centrifuged at 1500 rpm for 5 min and resuspended in 20 ml EGM. The cell suspension was divided equally between two 75 cm<sup>2</sup> tissue culture flasks. In general, it was possible to passage the cells once. Beyond passage number one, epithelial cells displayed a reduced growth rate and had difficulty adhering to the flasks.

#### 2.2.2c Fibroblast cell culture

The supernatant from 2.2.2a was centrifuged at 2000 rpm for 10 min and the resulting cell pellet was resuspended in 10 ml fibroblast growth medium (FGM; RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin). The fibroblast cell suspension was seeded into one 75 cm<sup>2</sup> flask and grown in monolayer culture at 37°C for 72 h. Following a change of fresh medium, the cells were maintained until 80% confluent. Sub-culture was performed as described in section 2.2.1a.

#### 2.2.3 Determination of cell numbers

##### 2.2.3a Manual determination of cell numbers by haemocytometer

Cells to be counted were washed in PBS, incubated with 1x trypsin-EDTA for 2 min at 37°C, centrifuged and resuspended in culture medium. Cell numbers were determined using both chambers of a haemocytometer with four of the 0.1 mm<sup>3</sup> squares in each chamber counted and an average of the counts taken.

### 2.2.3b Colorimetric determination of viable cell numbers

The proliferative rates of prostatic cells in primary culture were determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay according to the manufacturer's protocol (Promega). The MTS assay provides a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity studies (Maghni et al. 1999). MTS tetrazolium compound is bio-reduced by viable cells into a coloured formazan product that is soluble in tissue culture medium. Dead cells do not cause this change. The quantity of formazan product is measured spectrophotometrically at 490 nm and is directly proportional to the number of living cells in culture.

Cells were plated in 96 well plates, supplemented with 150-200 µl per well of the appropriate medium. At the end of an incubation period of 2-4 d of treatment with steroids or drugs, the culture medium was removed and 100 µl of MTS working solution (MTS stock solution diluted 1:5 in the appropriate culture medium) added. Cells were then incubated with MTS at 37°C for 1-3 h. Subsequent to this incubation, the absorbance of the resulting solution was measured at 490 nm with a microplate reader (model 450; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). At least 2 wells per plate containing no cells were incubated with the MTS working solution as blanks.

### 2.2.4 Measurement of the growth response of cultured cells

The growth response of epithelial cells and fibroblasts to oestrogenic compounds and the anti-oestrogen ICI 182780 (Tochris, Bristol, UK) was determined using the MTS assay as outlined in 2.2.3b. Whenever possible, media employed were phenol red-free and the FCS was rendered steroid hormone-free through



treatment with dextran-coated charcoal (DCC). All cells were grown in 96 well tissue culture plates.

#### 2.2.4a Preparation of DCC-stripped FCS (DCC-FCS)

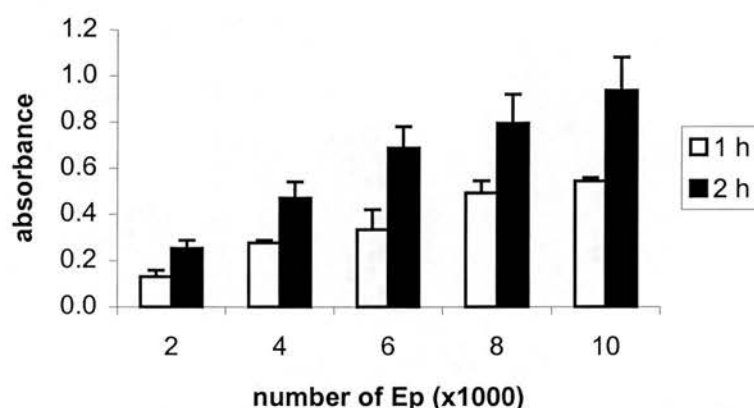
1 g DCC was mixed in an Erlenmeyer flask with 100 ml FCS and the mixture shaken at 4°C for 24 h. The charcoal was then removed by centrifugation and the serum filter-sterilised through a 0.2 µm filter. To test the efficiency of steroid stripping, 10 nM of each of the radiolabelled steroids [1,2,6,7-<sup>3</sup>H]-androstenedione, [1,2,6,7-<sup>3</sup>H]-testosterone, [4-<sup>14</sup>C]-dihydrotestosterone and [2,4,6,7-<sup>3</sup>H]-oestradiol was added to 1 of 4 flasks containing FCS. Aliquots of serum before and after DCC-stripping were added to 6 ml liquid gold scintillation cocktail (Canberra Packard, Pangbourne, UK). Radioactivity in each sample was then quantified using a Tri-Carb liquid scintillation counter (Canberra Packard). After DCC treatment, less than 5% of radioactivity remained in each sample of FCS compared with FCS before stripping.

#### 2.2.4b Preliminary MTS assays

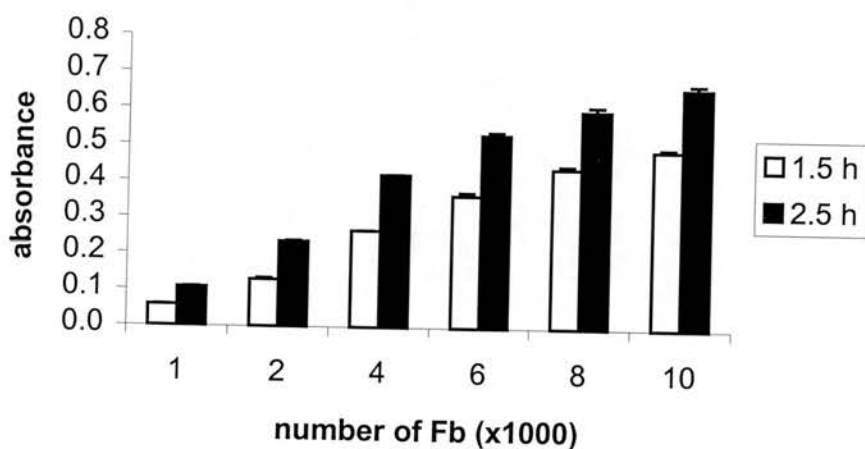
Preliminary experiments were carried out to confirm that the MTS assay is suitable for determining numbers of prostatic fibroblasts and epithelial cells in 96-well plates. Epithelial cells (passage number 1; fig. 2.1) and fibroblasts (passage number 2; fig. 2.2) were harvested as described above and plated in triplicate at a density of  $1-10 \times 10^3$  cells per well in 150 µl serum-free EGM and phenol red-free RPMI medium (PRF-RPMI) supplemented with 5% DCC-FCS respectively. 4 h after seeding, when cells have attached to the bottom of wells, the medium in each well was carefully removed and 100 µl MTS working solution was added. MTS assay was performed as outlined above in section 2.2.3b.



Figures 2.1 and 2.2 show that absorbance at 490 nm of the MTS solution in 96-well plates was proportional to the number of epithelial cells or fibroblasts seeded into each well up to approximately 8000 cells/well.



**Fig. 2.1 Measurement of epithelial cell number by the MTS assay.** Epithelial cells (Ep) were plated in triplicate in serum-free EGM at 2-10 x 1000 cells/well in a 96-well plate. 4 h after seeding, the medium in each well was removed and 100  $\mu$ l MTS working solution (MTS stock solution diluted 1:5 in serum-free EGM) was added to each well. 2 wells containing no cells were included as blanks. Cells were then incubated with MTS for 1 h (open bars) or 2 h (solid bars). Absorbance at 490 nm was measured using a microplate reader. Results are corrected by absorbance in control wells and expressed as mean of triplicates  $\pm$  s.e.m.



**Fig. 2.2 Measurement of fibroblast cell number by the MTS assay.** Fibroblasts (Fb) were plated in triplicate in PRF-RPMI supplemented with 5% DCC-FCS at 1-10 x 1000 cells/well in a 96-well plate. 4 h after seeding, the medium in each well was removed and 100  $\mu$ l MTS working solution (MTS stock solution diluted 1:5 in the same medium) was added to each well. 2 wells containing no cells were included as blanks. Cells were then incubated with MTS for 1.5 h (open bars) or 2.5 h (solid bars). Absorbance at 490 nm was measured using a microplate reader. Results are corrected by absorbance in control wells and expressed as mean of triplicates  $\pm$  s.e.m.

#### 2.2.4c Growth response experiments for epithelial cells and fibroblasts in primary culture

The growth of prostatic cells in primary culture over a period of 2-4 d was assessed by the MTS assay. Epithelial cells grown in monolayer culture in 75 cm<sup>2</sup> tissue culture flasks were harvested through trypsinisation as described before and the cell number was determined using a haemocytometer as described in section 2.2.3a. Cells were then resuspended in serum-free EGM to the desired final concentration and plated at a density of 3000 cells/well in 96-well plates. Fibroblasts were treated in the same manner except that the medium used was PRF-RPMI supplemented with 0-10% DCCFCS and the plating density was 1800 cells/well.

The final concentrations of oestrogens and anti-oestrogen in culture medium ranged from 1 nM to 10  $\mu$ M. 1 mM stock solutions of the compounds in ethanol were prepared and serially diluted down to 100 nM. For example, the addition of 20  $\mu$ l oestradiol (1  $\mu$ M) to 1.98 ml medium gave a final concentration of 10 nM. The final concentration of ethanol in medium did not exceed 1% (v/v). The medium in control wells was supplemented with 1% ethanol.

To investigate the effects of oestrogen/anti-oestrogen, epithelial cells in 96-well plates were grown in serum-free EGM and fibroblasts in PRF-RPMI supplemented with 2% or 10% DCC-FCS. 48 h before oestrogen/anti-oestrogen treatment, cells were plated in 0.15 ml/well of the appropriate medium (6 wells per treatment/control group) and transferred to a 37°C incubator. The remaining wells in the plates contained 0.18 ml PBS. 48 h later, the medium was carefully aspirated from the test wells and replaced with 0.18 ml of medium containing various concentrations of oestrogen/anti-oestrogen. The culture medium containing oestrogen/anti-oestrogen was changed every 24 h. Cell numbers were determined after 2 d of oestrogen/anti-oestrogen treatment using the MTS assay as described in section 2.2.3b.

## 2.3 Analysis of 5 $\alpha$ -reductase Activity in Tissue Homogenates

### 2.3.1 Preparation of BPH tissue homogenates

Although both isozymes of 5 $\alpha$ -reductase convert testosterone to dihydrotestosterone and androstenedione to 5 $\alpha$ -androstanedione, their differential pH optima was one of the first characteristics leading to the discovery of the two isozymes (Russell & Wilson 1994). The specific pH sodium phosphate buffers for 5 $\alpha$ -reductase isozyme assay were prepared as follows.

#### 5 $\alpha$ R2 buffer:

sodium phosphate (0.1M, pH 5.0)	1.5 ml x 1M Na <sub>2</sub> HPO <sub>4</sub>
	98.5 ml x 1M NaH <sub>2</sub> PO <sub>4</sub>
	diluted to 1000ml with distilled H <sub>2</sub> O

#### 5 $\alpha$ R1 buffer:

sodium phosphate (0.1M, pH 7.5)	81.5 ml x 1M Na <sub>2</sub> HPO <sub>4</sub>
	18.5ml x 1M NaH <sub>2</sub> PO <sub>4</sub>
	diluted to 1000ml with distilled H <sub>2</sub> O

Stock solutions of 0.1 M sodium phosphate buffer were diluted to 40 mM with distilled H<sub>2</sub>O and supplemented with 0.32 M sucrose.

Prostate tissue, obtained from men undergoing TURP operations for BPH, was snap frozen in liquid nitrogen and stored at -70°C. Approximately 300 mg frozen tissue was added to 6 ml sodium phosphate buffer (40 mM, pH 5.0 or 7.5) containing 0.32 M sucrose and 1 mM dithiothreitol (DTT). The tissue samples were then homogenised using a Ystral blender (3 x 15 s at 15,000 rpm). The homogenised tissue was then filtered through sterile gauze and transferred into Eppendorf tubes in 1 ml aliquots. The protein concentration of each aliquot was determined

colourimetrically using a Bio-Rad protein assay kit as described in section 2.3.4. Tissue homogenates were then prepared for 5 $\alpha$ -reductase assay or stored at -70°C.

### 2.3.2 5 $\alpha$ -reductase isozyme assay

The protocol was an adaptation of that described by Smith et al. (1996). Co-factor reagents were prepared as described below. All co-factors were aliquoted and stored at -20°C.

#### 10 mM NADP

255 mg dissolved in 30 ml sodium phosphate buffer (40 mM, pH 5.0 or 7.5)

#### 100 mM glucose-6-phosphate

1.144 g dissolved in 29 ml sodium phosphate buffer (40 mM, pH 5.0 or 7.5)

#### 5 units/ml glucose-6-phosphate dehydrogenase

100  $\mu$ l of 1 unit/ $\mu$ l glucose-6-phosphate dehydrogenase in 16 ml sodium phosphate buffer (40 mM, pH 5.0 or 7.5) and 4 ml glycerol

An NADPH-regenerating system (0.75 ml) consisting of 40 mM sodium phosphate buffer (pH 5.0 or 7.5), 0.32 M sucrose, 1 mM DTT, 0.5 mM NADP, 5 mM glucose-6-phosphate and 0.25 unit/ml glucose-6-phosphate dehydrogenase was incubated with 0.2 ml tissue homogenate and 10 nM <sup>3</sup>H-substrate (total volume of 1 ml) at 37°C for 0-60 min. Control reactions contained no tissue homogenate.

Reactions were stopped by the addition of 2 vol diethyl ether containing 1000 cpm each of <sup>14</sup>C-steroids and 25  $\mu$ g each of unlabelled androstenedione, testosterone, 5 $\alpha$ -androstenedione, dihydrotestosterone, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol and 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol. The resultant mixture was vortexed for 60 s. The assay tubes were then transferred to a -70°C freezer for 10-15 min to freeze the aqueous phase. The supernatant consisting of diethyl ether and extracted steroids was

decanted into clean glass test tubes and evaporated to dryness in a vacuum oven at 40°C. This extraction process was repeated once. After diethyl ether was evaporated, the residue was re-constituted in 50 µl ethanol and subjected to thin layer chromatography (TLC) as described in section 2.7.1a.

Loss of metabolite due to the extraction procedure was calculated by analysing the recovery of  $^{14}\text{C}$ -steroids from the samples.  $5\alpha$ -reductase activity was calculated by taking into consideration blank values, specific activities of radiolabelled steroids and loss due to the extraction process as follows.

$$\text{No. of moles of steroid metabolite formation} = \frac{\frac{\text{Hs} \times \text{Co}}{\text{Cs}} - \frac{\text{Hb} \times \text{Co}}{\text{Cb}}}{2.22 \times 10^{12} \times \text{S.A.}}$$

In the above formula, Hs and Hb are the  $^3\text{H}$  counts (dpm) of the sample and the blank respectively after TLC separation and scintillation counting. Cs and Cb are the  $^{14}\text{C}$  counts (dpm) of the sample and the blank respectively after TLC separation and scintillation counting. Co is the original  $^{14}\text{C}$  count (dpm) of the  $^{14}\text{C}$ -labelled steroid added to the assay for calculation of recovery. S.A. is specific activity of the  $^3\text{H}$ -labelled steroid (Ci/mol).

### 2.3.3 Preparation of [4- $^{14}\text{C}$ ]- $5\alpha$ -androstanedione

[4- $^{14}\text{C}$ ]- $5\alpha$ -androstanedione is not commercially available and was therefore synthesised in-house. Preparative reactions to synthesise [4- $^{14}\text{C}$ ]- $5\alpha$ -androstanedione were carried out under conditions identical to the  $5\alpha$ -reductase assay except that 1 µM [4- $^{14}\text{C}$ ]-androstenedione was incubated with BPH tissue homogenate for 24 h and no other steroid was added at the end of the incubation. The resultant radiolabelled  $5\alpha$ -androstanedione was purified by TLC as follows. The dried steroid was re-constituted in 50 µl ethanol and run in parallel to non-radiolabelled  $5\alpha$ -androstanedione on TLC plate as described in section 2.7.1a except that only the non-

radiolabelled standards were sprayed with a tracer and baked. The area of TLC plate containing [4-<sup>14</sup>C]-5 $\alpha$ -androstanedione was scraped off and eluted in diethyl ether, which was then evaporated to dryness in a vacuum oven at 40°C. The purity of the synthesised [4-<sup>14</sup>C]-5 $\alpha$ -androstanedione was confirmed by high performance liquid chromatography.

#### 2.3.4 Determination of protein concentration

Protein concentrations of tissue homogenates and cell lysates were determined colorimetrically using a protein assay kit (Bio-Rad DC Protein Assay Kit; Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's protocol, which is based on the method of Lowry (Lowry et al. 1951). A range of protein standards (0.1-5.0 mg/ml) was prepared using bovine serum albumin (BSA) in the appropriate buffer. 5  $\mu$ l protein sample or standard was transferred to a 96 well plate. To this sample, 25  $\mu$ l reagent A (an alkaline copper tartrate solution) and 200  $\mu$ l reagent B (containing Folin reagent) were added. The plate was then gently shaken on an orbital shaker for 15 min at room temperature. Subsequent to this incubation, the absorbance of the samples was read at 595 nm using a microplate reader (model 450; Bio-Rad laboratories, Hemel Hempstead, Hertfordshire, UK).

## 2.4 Preparation of Expression Plasmids

### 2.4.1 Plasmids for transfection studies

**pSV2L:** This is an expression plasmid containing a luciferase reporter gene driven by the simian virus 40 early promoter (De Wet et al. 1987). It was used as an internal standard for transfection efficiency.

**pCMV2** (GenBank accession no. AF239247): This 4.7 kb expression plasmid is a kind gift from Prof. Ian Mason (Clinical Biochemistry, University of Edinburgh)

and contains a simian virus 40 origin of replication, a cytomegalovirus promoter, and the human growth hormone termination and polyadenylation signals (Corbin et al. 1988).

**pCMV2.arom** (Corbin et al. 1988): This 6.6 kb plasmid encoding the human aromatase enzyme (also a gift of Prof. Ian Mason) was constructed by ligating a 1.9 kb cDNA fragment (encoding the full-length aromatase) between the *EcoRI* and *SmaI* restriction sites of the pCMV2 plasmid (Corbin et al. 1988).

**pCMV2.5 $\alpha$ R1**: This is a 5.5 kb expression plasmid constructed by ligating a 0.8 kb fragment of cDNA encoding human 5 $\alpha$ -reductase type 1 isozyme between the *BglII* and *HindIII* restriction sites of pCMV2 as described below in section 2.4.2.

#### 2.4.2 Subcloning of 5 $\alpha$ R1 cDNA

To construct a plasmid expressing human 5 $\alpha$ -reductase type 1 (5 $\alpha$ R1), a strategy similar to that used by Andersson & Russell (1990) was employed. The plasmid ph5 $\alpha$ 45 encoding full-length 5 $\alpha$ R1 (Andersson & Russell, 1990; a gift from Dr. David Russell, University of Texas) was used as a template to synthesise 5 $\alpha$ R1 cDNA. Two oligonucleotide primers derived from the 5' end of the cDNA

*BglII*  
(5'-ATAGATCTACCATGGCAACGGCGACGGGGGTGGCGG-3')

and corresponding to the 3' untranslated region

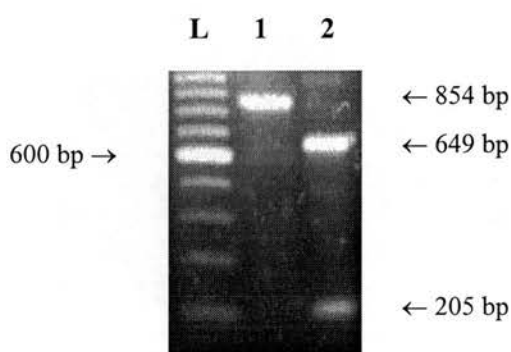
(5'-AAAGTCCATAGAGAAGCGCCATTGG-3')

were used in a polymerase chain reaction (PCR) with *PfuTurbo* DNA polymerase (Stratagene) to remove an upstream ATG and introduce a unique *BglII* restriction enzyme site in the 5' untranslated region.

The polymerase chain reaction consisted of denaturing at 94°C for 5 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min



and extension at 74°C for 1 min, with a final extension step at 74°C for 10 min. After amplification, the PCR product of 854 bp was isolated from a 1% low-melting-point agarose gel (Life Technologies, Paisley, UK) and purified with a DNA purification kit (Hybaid, Ashford, UK) according to the manufacturer's protocol. Identity of the purified PCR product was confirmed by the restriction enzyme *PvuII* as described in section 2.9.4 (fig. 2.3).



**Fig. 2.3 Restriction enzyme digestion of 5 $\alpha$ R1 PCR product.** The 854-bp 5 $\alpha$ R1 PCR product was digested with restriction enzyme *PvuII* to confirm its identity. *PvuII* produced two fragments of 649 and 205 bp. L, 100-bp DNA ladder; lane 1, 854 bp undigested PCR product; lane 2, *PvuII* digestion of PCR product.

The purified PCR product and the expression plasmid pCMV2 were cut with both *Bgl*III and *Hind*III restriction enzymes. These two restriction sites are located in the 5' and 3' untranslated regions of the 5 $\alpha$ R1 cDNA respectively. The 5 $\alpha$ R1 cDNA restriction fragment was then inserted between the *Bgl*III and *Hind*III restriction sites of pCMV2 in a 10  $\mu$ l ligation reaction according to the supplier's protocol (Promega). The ligation mixture containing 1  $\mu$ l of 10x ligation buffer (300 mM Tris-HCl pH 7.8, 100 mM MgCl<sub>2</sub>, 100 mM DTT, 10 mM ATP), 1 unit T4 DNA ligase (Promega) and 1:3 molar ratio of linearised pCMV2:cDNA fragment was incubated overnight at 4°C. The resultant pCMV2.5 $\alpha$ R1 was then used for bacterial transformation and plasmid DNA preparation ('miniprep') as described in section 2.4.3c.

### 2.4.3 Plasmid DNA preparation

#### 2.4.3a Bacterial media

Luria-Bertoni (LB) broth was prepared by dissolving 10 g tryptone (Difco), 5 g yeast extract (Difco) and 5 g sodium chloride in 1000 ml distilled water. For LB agar, 3.75 g agar was added to 250 ml LB broth. Ampicillin (Sigma) was added to LB broth at a concentration of 100  $\mu$ g/ml to select for plasmids with ampicillin resistance.

All media were sterilised by autoclaving. Bacteria were grown at 37°C in a shaking incubator to provide aeration.

#### 2.4.3b Bacterial transformation

*Escherichia coli* strain HB101 was made competent for DNA uptake by the calcium chloride method (Sambrook et al. 1989). Cells were grown in 50 ml LB to mid-log phase ( $A_{600} = 0.3-0.6$ ) and then harvested by centrifugation at 7000 rpm in a

Beckman J2-20 rotor for 5 min at 4°C. The pellet was resuspended in 20 ml ice-cold 0.1 M CaCl<sub>2</sub> and stored on ice for 10 min. Cells were then repelleted and resuspended in 2 ml ice-cold 0.1 M CaCl<sub>2</sub>. Competent cells could be stored at 4°C for up to 24 h before transformation.

200 µl competent cells were mixed with up to 50 ng plasmid DNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored on ice for 20-30 min. The cells were then heat-shocked at 42°C for 50 s and transferred back on ice for 2-3 min. The transformation mixture was spread onto an LB agar plate supplemented with 100 µg/ml ampicillin and was incubated at 37°C overnight. Control plates for bacterial transformation contained HB101 alone (which does not have the ampicillin resistance gene and thus acted as a negative control) or cells transformed by 10 ng of positive control plasmid, e.g. pGEM3. Cells that grew on the selective LB agar were successfully transformed by the recombinant DNA bearing the ampicillin resistance gene.

#### 2.4.3c Plasmid DNA miniprep (‘miniprep’)

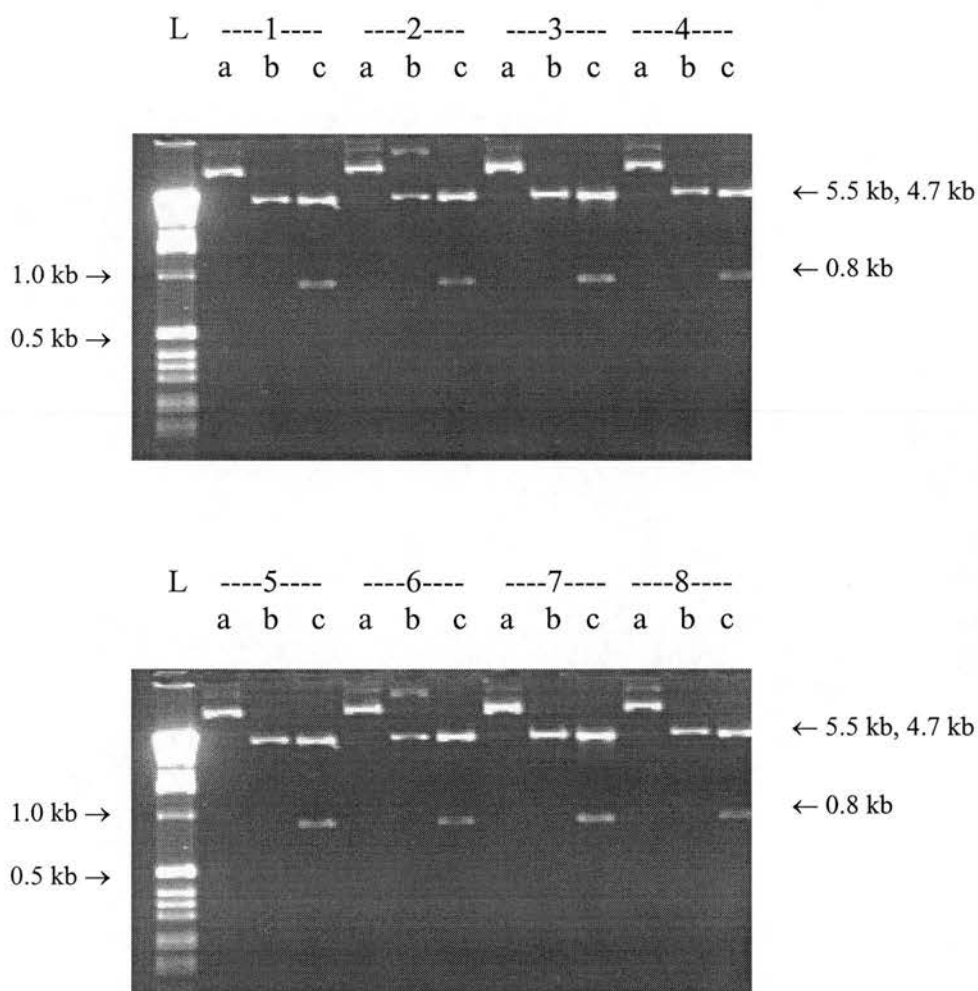
To screen the transformed cells for the presence of insert, 8 transformed bacterial colonies on the agar plates were transferred to 8 separate tubes containing 2 ml LB broth supplemented with 100 µg/ml ampicillin. The tubes were incubated overnight at 37°C in a shaking incubator. 1.5 ml of the overnight culture was recovered by centrifugation at 14,000 rpm in a bench top Eppendorf centrifuge for 1 min (the rest of the culture was stored at 4°C). The pellet was then resuspended in 100 µl ice-cold GTE (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA), mixed gently with 200 µl freshly prepared alkaline-SDS solution (0.2 M NaOH, 1% w/v SDS) and stored on ice for 15 min. This cell lysate was then mixed with ice-cold 150 µl potassium acetate (3 M with respect to potassium and 5 M with respect to acetate respectively, pH 4.8) and stored on ice for 15 min. Chromosomal DNA and cellular proteins were removed by centrifugation at 14,000 rpm for 5 min. 400 µl of the supernatant was transferred to a fresh Eppendorf tube and mixed with 400 µl

phenol:chloroform (1:1, v/v). After vortexing and centrifugation for 2 min, the supernatant was transferred to a fresh Eppendorf tube. DNA was then precipitated with 2 vol ethanol at room temperature for 5 min. After centrifuging for 5 min, the supernatant was removed. The DNA pellet was washed with 70% ethanol and dried at room temperature before resuspension in 50 µl TE buffer containing DNase-free RNase A.

8 samples of plasmid DNA obtained from the above 'miniprep' were then subjected to restriction enzyme digestion by *HindIII* and *BglIII* to confirm the presence of 5αR1 cDNA insert before large scale plasmid preparation (fig. 2.4).

#### 2.4.3d Large scale plasmid DNA preparation ('Maxiprep')

2 ml LB broth supplemented with 100 µg/ml ampicillin was inoculated with a single bacterial colony which contained the required plasmid and incubated overnight at 37°C. The overnight culture was diluted in 500 ml LB broth supplemented with 100 µg/ml ampicillin, grown in a 2 litre flask at 37°C overnight and centrifuged at 6000 rpm for 5 min at 4°C in a Beckman J2-20 rotor. The bacterial pellet was then resuspended in 12 ml ice-cold GTE solution (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA), mixed immediately with 24 ml freshly prepared alkaline-SDS solution (0.2 M NaOH, 1% w/v SDS) and stored on ice for 5 min. 16 ml ice-cold potassium acetate (3 M with respect to potassium and 5M with respect to acetate respectively, pH 4.8) was added, mixed and stored on ice for 10 min before centrifuging at 6000 rpm for 10 min at 4°C. The supernatant was filtered through 4 layers of sterile gauze into 250 ml centrifuge pots, and precipitated at room temperature for 30 min by the addition of 32 ml isopropanol. Plasmid DNA was recovered by centrifuging at 10,000 rpm for 3 min. The supernatant was removed and the pellet dried at room temperature before resuspension in 2.2 ml TE buffer.



**Fig. 2.4 Restriction enzyme digestion of the plasmid pCMV2.5 $\alpha$ R1.** 8 samples (numbered 1-8) of the plasmid pCMV2.5 $\alpha$ R1 obtained from miniprep (section 2.4.3c) were digested with *Hind*III and *Bgl*II to confirm their identity. L, DNA ladder; (a), uncut pCMV2.5 $\alpha$ R1; (b) plasmid digested with *Hind*III to produce linearised DNA (5.5 kb); (c) plasmid digested with both *Hind*III and *Bgl*II to produce 2 fragments of 4.7 kb and 0.8 kb.

1 g caesium chloride (CsCl) was added to each ml of DNA solution. 50  $\mu$ l ethidium bromide (10 mg/ml) was then added. The resulting DNA/CsCl solution was transferred to a 3 ml Beckman Quick Seal ultracentrifuge tube with enough CsCl solution (prepared by adding 100 g CsCl to 100 ml TE buffer) to fill the tube and centrifuged at 70,000 rpm for 16 h or 100,000 rpm for 4 h at 20°C in a Beckman Optima LTX Ultracentrifuge. The plasmid DNA band was collected through the seal tube wall using a gauge 21 needle coupled to a syringe. The plasmid was then transferred to a fresh seal tube with enough CsCl solution to fill the tube, and centrifuged again at 70,000 rpm for 16 h or 100,000 rpm for 4 h at 20°C. The plasmid DNA band was again collected through the wall of the seal tube.

The ethidium bromide was removed from the CsCl solution by repeated extraction with isopropanol until the pink colour disappeared from the CsCl-DNA solution. Plasmid DNA was purified from CsCl by dialysis in Visking dialysis tubing (molecular weight cut-off 12-14 kDa; Medicell, London, UK) against 3 changes of 2 l TE buffer at 4°C for 4 h or overnight. DNA concentration of the resultant plasmid was determined spectrophotometrically at 260 nm (section 2.8.1) and stored at -20°C.

After large scale plasmid DNA preparation, pCMV2.5 $\alpha$ R1 was fully sequenced by Mr. Stewart McKay (Human Genetics Unit, Medical Research Council, Edinburgh) using the ABI PRISM<sup>®</sup> sequencing kit (Applied Biosystems, Warrington, UK) to verify its authenticity.

## 2.5 Transfection Studies

### 2.5.1 Screening of cell lines for endogenous enzyme activities

CHO, COS-1 and C6 cells were screened for their endogenous  $5\alpha$ -reductase and aromatase activities. On day 1 of experiment, cells were harvested by trypsinisation.  $3 \times 10^5$  cells were plated in 4 ml DMEM with 10% DCCFCS in 60 mm culture dishes and allowed to adhere overnight in a  $37^\circ\text{C}$  incubator. The medium was replaced by 4 ml of the same medium the next day. On day 3, the medium was replaced with 4 ml of the same medium containing 10 nM  $^3\text{H}$ -androstenedione or  $^3\text{H}$ -testosterone. After 4, 6 or 24 h of incubation, the medium was collected into a 20 ml glass vial (Packard Biosciences, Pangbourne, UK) and stored at  $-20^\circ\text{C}$ . Steroids in the medium were extracted with 5 volumes of HPLC grade ethyl acetate and dried under a nitrogen gas stream at  $45^\circ\text{C}$ . The residue was then resuspended in 1 ml HPLC mobile phase (50% water, 35% acetonitrile and 15% methanol) and analysed by an HPLC system (section 2.7.2), which had been optimised to separate testosterone, androstenedione, oestradiol, oestrone,  $5\alpha$ -dihydrotestosterone,  $5\alpha$ -androstanedione and androsterone.

### 2.5.2 Solutions and buffers for transfection

The compositions of solutions and buffers employed in transfection experiments are as follows.

2x HBS (HEPES-buffered saline)	50 mM HEPES 280 mM NaCl 1.5 mM $\text{Na}_2\text{HPO}_4$ , pH 7.05 filter-sterilised through 0.2 $\mu\text{m}$ filter
Calcium chloride ( $\text{CaCl}_2$ ) 2 M	filter-sterilised through 0.2 $\mu\text{m}$ filter



Lysis buffer X	25 mM Tris-phosphate, pH 7.8
	2 mM DTT
	1% Triton X-100
	10% glycerol
Luciferase assay buffer	40 mM tricine
	67 mM DTT
	0.2 mM EDTA
	magnesium carbonate (48.6 mg/ml)
	magnesium sulphate (0.64 mg/ml)
	co-enzyme A (0.2 mg/ml)

### 2.5.3 Transient transfection by the calcium phosphate method

Cells were transiently transfected using the calcium phosphate coprecipitation method as described (Sambrook et al. 1989) with minor modifications. Exponentially growing cells were harvested by trypsinisation.  $2-3 \times 10^5$  cells were plated in 4 ml DMEM supplemented with 10% DCC-FCS in 60 mm culture dishes and the cultures were incubated for 20-24 h at 37°C in a humidified incubator. The next day, 4 ml of the same medium was replaced at least 1 h before transfection. 300 µl solution containing 37 µl  $\text{CaCl}_2$  (2 M) and a total of 15 µg plasmid DNA was added dropwise to an equal volume of 2x HBS with gentle vortexing. The calcium phosphate-DNA coprecipitate was allowed to form at room temperature for 15 min. The calcium phosphate-DNA suspension was then transferred drop by drop into the medium above the cell monolayer and the cells were incubated at 37°C for 20-24 h. On day 3 of experiment, the medium and precipitate were removed by aspiration and washed once with serum-free DMEM. Each culture dish was then covered with 3 ml DMEM containing 10% DCC-FCS and returned to the incubator at least 1 h before steroid/drug treatment. Steroids/drugs in 1 ml medium were then added to the monolayer cell culture and incubated for 2-6 h. The medium was subsequently



collected into a 20 ml glass vial and stored at  $-20^{\circ}\text{C}$  until steroid extraction. Steroids were extracted from the culture medium with 5 vol ethyl acetate, dried at  $45^{\circ}\text{C}$  under a nitrogen gas stream and stored at  $-20^{\circ}\text{C}$  until high performance liquid chromatography (HPLC) analysis. After removal of culture medium, the cell monolayer was washed twice with 3 ml PBS and lysed with 300  $\mu\text{l}$  lysis buffer X for 10-15 min at room temperature. Cells from each dish were then scraped, transferred to an Eppendorf tube and centrifuged at 14,000 for 2 min in a bench top microcentrifuge. An aliquot of the supernatant was used immediately for luciferase assay.

#### 2.5.4 Luciferase assay

The firefly luciferase catalyses the ATP-dependent conversion of luciferin to oxyluciferin with light emission. 100  $\mu\text{l}$  luciferase assay buffer was mixed with 5  $\mu\text{l}$  ATP (0.1 M, pH 7) and 40  $\mu\text{l}$  cell lysate containing luciferase. Cell lysate prepared from COS-1 cells transfected with pCMV2 alone was included in every assay to give a background reading of luciferase activity. The reaction mixture was placed in the counting chamber of a single-tube luminometer coupled with injector (Lumat LB9501, Berthold) and 105  $\mu\text{l}$  beetle luciferin (1 mM, Promega) was injected to the reaction mixture. An instantaneous measurement of light emission was then recorded.

## 2.6 Steroid Metabolism in Prostatic Cells in Primary Culture

### 2.6.1 Androstenedione metabolism in epithelial cells and fibroblasts

After the establishment of a relationship between  $5\alpha$ -reductase type 1 isozyme and aromatase in the co-transfection model, interaction between the two enzymes was further investigated in prostatic fibroblasts and epithelial cells in primary culture. On day 1 of each experiment, fibroblasts (passage number 2 or 3) in

2 ml phenol red-free RPMI medium supplemented with 10% DCCFCS were seeded in 6-well plates at a density of  $3 \times 10^5$  cells/well. The medium in each well was replaced by the same medium the following day. 24 h later, cells were incubated with 2 ml of the same medium containing 20 nM [ $^3\text{H}$ ]-androstenedione for 2-52 h. 2 wells with no cells were included in each experiment as controls to detect any non-enzymatic degradation of steroids. Epithelial cells (passage number 1) were treated in the same manner except that  $2 \times 10^5$  cells were incubated in 2 ml serum-free EGM per well for up to 24 h because of their high rates of substrate metabolism. The culture medium was then transferred to a 20 ml glass vial and  $^{14}\text{C}$ -steroids (500-1000 cpm each) added for determination of recovery after extraction. Steroids were extracted with 5 vol ethyl acetate and dried under a nitrogen gas stream at  $45^\circ\text{C}$ . After collection of medium, the remaining cells were washed twice with 3 ml PBS and lysed with 300  $\mu\text{l}$  lysis buffer X (25 mM Tris phosphate pH 7.8, 2 mM DTT, 1% Triton X-100 and 10% glycerol). Determination of cell lysate protein concentration was as described in section 2.3.4. Steroids were initially dissolved in 1 ml mobile phase (50% water, 35% acetonitrile and 15% methanol) to identify all metabolites by HPLC. Because of the small amount of oestrone formed, a two-step thin layer chromatography system was employed later to isolate oestrone from  $5\alpha$ -androstenedione and other steroids (section 2.7.1b).

## 2.6.2 Oestrogen metabolism in epithelial cells and fibroblasts

The metabolism of oestradiol and oestrone was examined in prostatic fibroblasts and epithelial cells. Fibroblasts (passage number 2 or 3) were seeded in 6-well plates at  $50\text{-}200 \times 10^3$  cells/well in 2 ml PRF-RPMI medium with 10% DCC-FCS. Epithelial cells (passage number 1) were seeded in 6-well plates at  $50\text{-}75 \times 10^3$  cells/well in 2 ml serum-free EGM. 2 control wells in each experiment contained no cells but were treated otherwise in the same manner as test wells. After 48 h, cells were incubated with 2 ml fresh medium containing 10 nM of either [ $^3\text{H}$ ]-oestrone or [ $^3\text{H}$ ]-oestradiol for 2-24 h. Preliminary experiments were carried out to establish the number of cells and incubation time required to achieve linear rates of oestrogen

metabolism (section 4.4). In subsequent experiments, culture medium was collected after 4 h of incubation with substrate and transferred to 20 ml glass vials to be stored at  $-20^{\circ}\text{C}$  until steroid extraction. At the end of the incubation period, the monolayer cell culture was washed twice with 3 ml PBS and lysed with 300  $\mu\text{l}$  lysis buffer X (section 2.5.2) for determination of protein concentration as described in section 2.3.4. Steroids were extracted from culture medium with 5 vol ethyl acetate and dried under a nitrogen gas stream. The residue was reconstituted in mobile phase (water 50%, acetonitrile 35%, methanol 15%) and analysed by reversed-phase high performance liquid chromatography.

## **2.7 Analysis of Steroid Metabolites**

### **2.7.1 Thin layer chromatography (TLC)**

#### **2.7.1a Separation of $5\alpha$ -reductase metabolites by TLC**

Following evaporation to dryness, products of  $5\alpha$ -reductase isozyme assay were resuspended in 50  $\mu\text{l}$  ethanol. The resuspended extract was spotted onto ITLC<sup>TM</sup> SA polysilica acid gel impregnated glass fibre TLC plates (Gelman Sciences) and separated by TLC with a mobile phase of dichloromethane:diethyl ether (9:1, v/v) in a glass chromatography tank for approximately 40 min, or until the solvent front reached the boundary of the TLC plate. Separated steroids were then visualised by spraying the TLC plates with 10% phosphomolybdic acid and baking at  $90^{\circ}\text{C}$  for colour development. Relative positions of steroids on TLC plate were as previously described by Houston et al. (1985). Areas of TLC plates corresponding to various steroids run alongside the steroid standards were removed by scraping the appropriate area into scintillation vials with a clean scalpel. 6 ml liquid gold scintillation cocktail (Canberra Packard, Pangbourne, UK) was added to each scintillation vial and radioactivity was quantified using a Tri-Carb liquid scintillation

counter (Canberra Packard).  $5\alpha$ -reductase activity was calculated, taking into consideration recovery, blank values and specific activities of radiolabelled steroids.

#### 2.7.1b Two-step TLC for the separation of steroid metabolites from primary prostatic cell cultures

A two-step TLC system was employed to isolate oestrone ( $E_1$ ) from  $5\alpha$ -androstanedione ( $5\alpha A$ ) and other steroids. For the first TLC run, the residue was reconstituted in 60  $\mu$ l ethanol:ethyl acetate (50:50, v/v) and developed in dichloromethane:diethyl ether (9:1, v/v) on ITLC<sup>TM</sup> SA polysilica acid gel impregnated glass fibre TLC plates (Gelman Sciences) alongside steroid standards. Under these conditions,  $5\alpha A$  was clearly separated from androstenedione and all other metabolites, except  $E_1$ . In order to separate  $E_1$  from  $5\alpha A$ , areas of TLC plate corresponding to  $E_1$  and  $5\alpha A$  were scraped off, eluted with ethyl acetate, dried and reconstituted as above. The second TLC run consisted of a mobile phase of benzene:ethanol (40:1, v/v). Areas of  $5\alpha A$  and  $E_1$  were visualised by spraying the TLC plates with 10% phosphomolybdic acid and baking at 90°C for colour development, scraped off and quantified using a Tri-Carb liquid scintillation counter.  $R_f$  values of  $5\alpha A$  and  $E_1$  in the second TLC run were 0.70 and 0.55 respectively. Identities of steroid eluant from TLC scrapings were further confirmed by reversed-phase HPLC.

#### 2.7.2 High performance liquid chromatography (HPLC)

Dried steroids were resuspended in 1 ml mobile phase (50% water, 35% acetonitrile and 15% methanol) and 200  $\mu$ l was injected into the high performance liquid chromatography (HPLC) system. The HPLC system consists of a Waters (Watford, UK) auto-injector, mobile phase pump, reversed-phase Symmetry<sup>®</sup> C8 column (4.6 x 150 mm, particle size 5  $\mu$ m; Waters), a column heater and a Berthold radioactivity monitor linked to a scintillation fluid pump. The system is controlled by computer software Winflow (JMBS Developments, Fontanil, France). The flow

rates of the mobile phase and Quicksafe Flow-2<sup>®</sup> scintillant (Zinsser Analytic, Maidenhead, UK) were set at 0.7-1.0 ml/min and 2.1-3.0 ml/min respectively to achieve optimal counting. The column heater was set at 35°C to maintain stability of retention times. Radioactive standards were injected at the start of every batch of samples to confirm the identities of the peaks. The area under each peak was integrated using the Winflow software and compared with the total area under all peaks. Routinely, blank incubations with no cells or tissues were performed for each experiment to detect any degradation of radiolabelled steroids.

The temperature of column, composition of HPLC mobile phase and pump rate were systematically optimised. At 35°C and mobile phase (50% water, 35% acetonitrile and 15% methanol) at a flow rate of 0.7 ml/min, retention times (min) of the following steroids were: oestradiol (10.18), testosterone (12.95), oestrone (15.01), androstenedione (16.34), 5 $\alpha$ -dihydrotestosterone (19.95), 5 $\alpha$ -androstanedione (22.14) and androsterone (29.70).

## **2.8 Isolation of Ribonucleic Acids from Cell Cultures**

Total RNA was extracted from cultured cells using either a modification of the method of Chomczynski & Sacchi (1987) or the TRI reagent (Sigma) according to the manufacturer's protocol. In order to minimise RNase contamination of the RNA preparation, all solutions were prepared using RNase-free glassware, plasticware, water and chemicals. Wherever possible, the solutions were treated with 0.1% DEPC (diethyl pyrocarbonate) for 24 h at room temperature and subsequently autoclaved.

### **2.8.1 Guanidium thiocyanate (GTC) method**

The GTC method is based on the one described by Chomczynski & Sacchi (1987). GTC denaturing solution (20 ml) was prepared by dissolving 9.46 g guanidine isothiocyanate in a mixture of 11.08 ml DEPC-treated H<sub>2</sub>O and 636  $\mu$ l

sodium citrate (0.75 M, pH 7.0), followed by the addition of 144  $\mu$ l 2-mercaptoethanol.

Cells were grown in monolayer culture to 80% confluence in 75 cm<sup>2</sup> tissue culture flasks, washed twice in DEPC-treated PBS and lysed in 1.9 ml GTC denaturing solution per flask. 100  $\mu$ l n-lauryl sarcosine (10%) was then added to each flask and mixed by gentle agitation. The tissue culture flask was shaken at 4°C for 10 min to ensure complete lysis of the cells. 500  $\mu$ l aliquots of the cell lysate were pipetted into Eppendorf tubes and 50  $\mu$ l sodium acetate (2 M, pH 4.0) was added to each tube. The tubes were vortexed for 10 s prior to the addition of 500  $\mu$ l water-saturated phenol and 100  $\mu$ l chloroform-isoamyl alcohol (49:1, v/v). The resulting suspension was vortexed for 10 s and then cooled on ice for 15 min. The samples were centrifuged at 13,000 rpm for 20 min at 4°C in a microcentrifuge. Subsequently, the upper aqueous phase was transferred to a fresh Eppendorf tube where it was mixed with an equal volume of isopropanol. The RNA was precipitated overnight at -20°C. The RNA precipitate was sedimented by further centrifugation at 13,000 rpm for 20 min at 4°C. The RNA pellet was subsequently resuspended in 60  $\mu$ l GTC denaturing solution. The samples were pooled and precipitated with an equal volume of isopropanol at -20°C for 2 h. The RNA was centrifuged at 13,000 rpm for 20 min at 4°C and the resulting pellet was washed in 200  $\mu$ l ethanol (75%) prior to a further 10 min centrifugation. The supernatant was carefully aspirated and the RNA allowed to air dry at room temperature before resuspension in 50  $\mu$ l nuclease-free water.

A 5  $\mu$ l aliquot of the RNA solution was diluted in 1.495 ml of DEPC-treated water and the concentration and purity of the preparation determined spectrophotometrically. In order to quantify the amount of RNA in the sample, readings were taken at 260 nm and 280 nm. An OD<sub>260</sub> of 1 corresponds to an RNA concentration of approximately 40  $\mu$ g/ml (Sambrook et al. 1984). The ratio between readings taken at 260 nm and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>) provides an estimate of the purity of the sample. A ratio of 2 corresponds to a pure sample. If the OD<sub>260</sub>/OD<sub>280</sub>



ratio was less than 1.7, the sample was re-extracted with phenol-chloroform as follows. 45  $\mu$ l phenol:chloroform (1:1) was added to the RNA solution and the suspension vortexed and centrifuged at 13,000 rpm for 5 min at 4°C. The aqueous phase was transferred to a fresh Eppendorf tube and mixed with 45  $\mu$ l chloroform:isoamyl alcohol (49:1) prior to further centrifugation at 13,000 rpm for 5 min at 4°C. The aqueous phase was removed and the RNA precipitated by the addition of 3 vol ethanol and 3 M sodium acetate to 0.3 M final concentration. The precipitated RNA was pelleted and resuspended in 50  $\mu$ l nuclease-free water. The concentration and purity of the RNA solution was then re-assessed spectrophotometrically as before.

### 2.8.2 TRI reagent method

TRI reagent (Sigma) was used later in the project because it involved less steps and time than the traditional GTC method. The reagent is a mixture of guanidine thiocyanate and phenol in a mono-phase solution. The procedure is an improvement of the method of Chomczynski & Sacchi (1987).

Cells were grown in monolayer culture to 80% confluence in 75 cm<sup>2</sup> tissue culture flasks, washed twice in DEPC-treated PBS and lysed with 2 ml TRI reagent at room temperature for 5 min. The cell lysate was passed several times through a pipette to form a homogenous lysate, 1 ml aliquot of which was then transferred to each of two Eppendorf tubes. 0.2 ml chloroform was added to each Eppendorf tube. The mixture was shaken vigorously for 15 s and allowed to stand at room temperature for 10 min before centrifugation at 13,000 rpm for 15 min at 4°C. Centrifugation separated the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and an uppermost colourless aqueous phase (containing RNA).

The aqueous phase was carefully transferred to a fresh Eppendorf tube and the RNA was precipitated by 0.5 ml isopropanol per ml of TRI reagent used in

sample preparation at room temperature for 10 min. The RNA precipitate was subsequently pelleted by centrifugation at 13,000 rpm at 4°C for 10 min. The supernatant was removed and the pellet washed with 1 ml of 75% ethanol. The sample was then vortexed and centrifuged at 6,500 rpm at 4°C for 5 min. The supernatant was carefully aspirated and the RNA allowed to air dry at room temperature before resuspension in 50 µl nuclease-free water. Concentration of RNA and purity of samples were determined spectrophotometrically as described before.

## **2.9 Analysis of Nucleic Acids**

### **2.9.1 Reverse transcription of total RNA**

The protocol for first strand synthesis of cDNA from total RNA is a modification of that described in the Promega Protocols and Applications Guide (3<sup>rd</sup> Edition, 1996). A volume of total RNA solution corresponding to 1 µg total RNA was aliquoted into a 500 µl microcentrifuge tube. The following solutions (all from Promega) were added to the RNA solution:

0.5 µl of rRNasin ribonuclease inhibitor (40 units/µl)

2 µl of 10 mM dNTP mixture

2 µl of 10x reverse transcription buffer containing

100 mM Tris-HCl (pH 9.0), 500 mM KCl and 1% Triton X-100

4 µl of 25 mM MgCl<sub>2</sub>

1 µl of oligo(dT)<sub>15</sub> (0.5 µg/µl)

0.75 µl of AMV reverse transcriptase (20 units/µl)

Made up to a final volume of 20 µl with nuclease-free H<sub>2</sub>O

The tube was vortexed, pulsed in a microcentrifuge and then incubated at 42°C for 60 min. Following the incubation, the reverse transcription mixture was heated to 99°C for 5 min, followed by incubation on ice for 5 min.



## 2.9.2 Polymerase chain reaction

Target mRNA	Name of primer	Primer sequence (5'→3')	PCR size (bp)	Source of primer sequence
Aromatase	Arom-2b	CCTCTGAGGTCAAGGAACAC	294	Harada et al. (1993)
	Arom-3	CAGAGATCCAGACTCGCATG		Dr. Forbes Howie*
ER $\alpha$	ER $\alpha$ -1	TACTGCATCAGATCCAAGGG	650	Lau et al. (1999)
	ER $\alpha$ -2	ATCAATGGTGCCTGGTTGG		
ER $\beta$	ER $\beta$ F	GTTGCGCCAGCCCTGTTAC	317	Dr. Colin Bayne <sup>+</sup>
	ER $\beta$ R	CTCGTCGGCACTTCTCTGTCTC		
GAPDH	GAPDHF	CCACCCATGGCAAATTCCATGGCA	598	Lau et al. (1999)
	GAPDHR	TCTAGACGGCAGGTCAGGTCCACC		

Table 2.1 Sequences of primers for polymerase chain reaction (PCR). All primer sequences are written 5' to 3'. PCR, polymerase chain reaction product. \*Dr. Forbes Howie, Clinical Biochemistry, Royal Infirmary, University of Edinburgh. <sup>+</sup>Dr. Colin Bayne, Breast Unit Research Group, Western General Hospital, University of Edinburgh.

The procedure for the amplification of cDNA sequences was based on that described in the Promega Protocols and Applications Guide (3<sup>rd</sup> Edition, 1996). The reaction mixture consisted of the following.

10  $\mu$ l of reverse transcription reaction

5  $\mu$ l of upstream primer (4  $\mu$ M)

5  $\mu$ l of downstream primer (4  $\mu$ M)

4  $\mu$ l of 10x reaction buffer containing

100 mM Tris-HCl (pH 9.0), 500 mM KCl and 1% Triton X-100

4  $\mu$ l of MgCl<sub>2</sub> (25 mM)

1  $\mu$ l of dNTP mix (10 mM)

1.25 units of Taq DNA polymerase

Made up to a final volume of 50  $\mu$ l with nuclease-free water

10 % (v/v) dimethyl sulphoxide (DMSO) was also included in the PCR of ER $\alpha$  and ER $\beta$  to improve the yield of PCR product. The components of the reaction mixture were thoroughly mixed by brief vortexing. The mixture was overlaid with 50  $\mu$ l mineral oil and pulsed in a microcentrifuge. PCR cycles consisted of 35-40 cycles (denaturation at 94°C for 1 min, annealing at 55-58°C for 1 min and extension at 72°C for 1 min) followed by a final extension step at 72°C for 10 min.

### 2.9.3 Agarose gel electrophoresis of DNA

PCR products were separated by electrophoresis on 1.5% agarose gels containing 0.5x Tris-borate-EDTA (TBE) buffer. 5x TBE buffer was prepared as follows.

5x TBE buffer	54 g Tris-base
	27.5 g boric acid
	10 ml EDTA (5 M, pH8.0)
	distilled H <sub>2</sub> O to a final volume of 1 litre

#### 2.9.3a Preparation of agarose gels

1.5% agarose gel was prepared by melting 1.5 g agarose NA in 100 ml 0.5x TBE in a microwave oven. The molten agarose was cooled to approximately 40°C before the addition of 1  $\mu$ l ethidium bromide (10 mg/ml). The agarose was then poured into a level tray containing a 16-well comb of well dimensions 5.5x1.5 mm and allowed to set at room temperature.

#### 2.9.3b Electrophoresis of samples in agarose gels

The gel was run in 800 ml 0.5x TBE in midi-gel apparatus (Northumbria Biologicals). 18  $\mu$ l of each PCR reaction with 3  $\mu$ l of DNA loading buffer (0.25% bromophenol blue and 30% glycerol, v/v) could be loaded per well alongside 20  $\mu$ l

of 100 bp ladder (approx. 0.1 mg/ml). Electrophoresis was performed for 2 h at 75 V (or as long as required to see sufficient separation of DNA fragments) after which the gel was examined and photographed under ultraviolet illumination.

2.9.4 Restriction enzyme digest

PCR products were cut by restriction enzymes to confirm their identities. The protocol is based on that described in the Promega Protocols and Applications Guide (3<sup>rd</sup> Edition, 1996). The restriction digest reaction consisted of the following.

Nuclease-free water	
Restriction enzyme 10x buffer	2 µl
PCR product	5-10 µl
Restriction enzyme (2-10 units)	1 µl
Final volume	20 µl

The mixture was incubated at 37°C for 2 h. 15 µl of the digested PCR product was then mixed with 3 µl DNA loading buffer and analysed by gel electrophoresis as described in section 2.9.3b. Sizes of the DNA fragments produced by various restriction enzymes are listed in table 2.2.

PCR product	Size of PCR product (bp)	Restriction enzyme	Size of DNA fragments (bp)
5αR1	854	<i>PvuII</i>	205, 649
aromatase	294	<i>AvaII</i>	102, 192
ERα	650	<i>PvuII</i>	308, 342
		<i>PstI</i>	325 (x2)
ERβ	317	<i>DraI</i>	125, 192
		<i>HinfI</i>	91, 226

Table 2.2 Restriction enzymes for identification of PCR products and predicted sizes of DNA fragments.

## **2.10 Preparation of Permixon®**

Permixon® was supplied as an n-hexane extract by Pierre-Fabre Medicament (Castres, France). The hexane was dried under a nitrogen gas stream at room temperature and the remaining residue dissolved in 10 ml ethanol to give a stock concentration of 10 mg/ml. Serial dilution of this stock solution was made in ethanol. Two different batches (batch numbers 735 and 762) were tested in all experiments involving Permixon®. Final concentration of ethanol in culture medium never exceeded 1%.

## **2.11 Statistical Analysis**

To determine if the means of two control/test groups were significantly different, statistics were carried out using the unpaired Student's *t*-test, unless otherwise specified, to calculate the *P* value. *P* value of <0.05 is considered statistically significant. Results are expressed as mean  $\pm$  standard error of mean (s.e.m.).

## **Chapter 3**

### **Results:**

#### **Interaction between**

#### **5 $\alpha$ -Reductase Type 1 Isozyme and Aromatase**

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- 3.1 5 $\alpha$ -reductase isozyme activity in BPH tissue homogenates
- 3.2 Establishment of a co-transfection model
- 3.3 Interaction between 5 $\alpha$ R1 and aromatase in co-transfection model
- 3.4 Aromatase mRNA expression in prostatic cells in primary culture
- 3.5 Androstenedione metabolism in prostatic cells in primary culture
- 3.6 Interaction between 5 $\alpha$ R1 and aromatase in prostatic cells in primary culture
- 3.7 Discussion

The role of 5 $\alpha$ R2 in the maintenance of normal prostatic growth and function is well established (section 1.3.2). In contrast, the function of 5 $\alpha$ R1 in the prostate is still unclear. To determine if 5 $\alpha$ R1 is a suitable target for therapeutic intervention, its function in the prostate needs to be clarified. Substrates for 5 $\alpha$ R1 are also substrates for aromatase but products of 5 $\alpha$ R1 activity are non-aromatisable steroids (section 1.4.1). Recent work in female mice suggested a key role for 5 $\alpha$ R1 during pregnancy in directing androgen substrates away from oestrogen synthesis by the enzyme aromatase in the utero-placental unit (Mahendroo et al. 1997). In the present study, the hypothesis that 5 $\alpha$ R1 plays a role in the regulation of aromatase activity in man by limiting substrate availability to the latter was tested first in a co-transfection model and then in human prostatic cells in primary culture. As the expression of aromatase in the human prostate is still controversial, this issue was also examined.

### **3.1 5 $\alpha$ -Reductase Isozyme Activity in BPH Tissue Homogenates**

Before investigating the role of 5 $\alpha$ R1 in androgen metabolism, it was necessary to determine which of the two androgens is a better substrate for 5 $\alpha$ R1. Previous kinetic studies demonstrated that human 5 $\alpha$ R1 expressed in CHO cells displayed higher affinity and catalytic efficiency with androstenedione than with testosterone (table 1.2, p.16; Thigpen et al. 1993, Levy et al. 1995). Moreover, androstenedione concentrations are higher than those of testosterone within the human prostate, whereas the reverse is true in plasma (Habib et al. 1976). To establish if androstenedione is the more metabolically relevant substrate for 5 $\alpha$ R1 in the human prostate, 5 $\alpha$ -reductase conversion of the two androgens at physiological concentrations was therefore examined in prostate tissue homogenates.

### 3.1.1 Preliminary 5 $\alpha$ -reductase assays

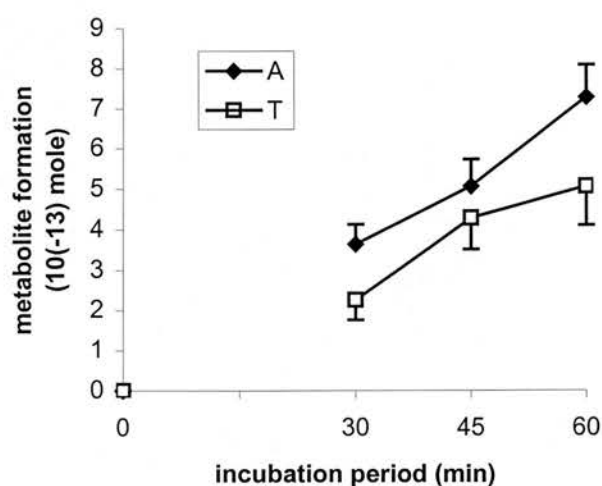
Preliminary experiments were carried out for the 5 $\alpha$ -reductase assays with both androstenedione and testosterone using tissue homogenates prepared from two BPH patients. For both 5 $\alpha$ -reductase isozymes, the conversion of androstenedione to 5 $\alpha$ A and that of testosterone to DHT was essentially proportional with time within 45 min incubation (fig. 3.1). Subsequent assays were therefore incubated for 30 min, using the same substrate concentrations and amount of tissue homogenates.

### 3.1.2 Comparison of androstenedione and testosterone metabolism

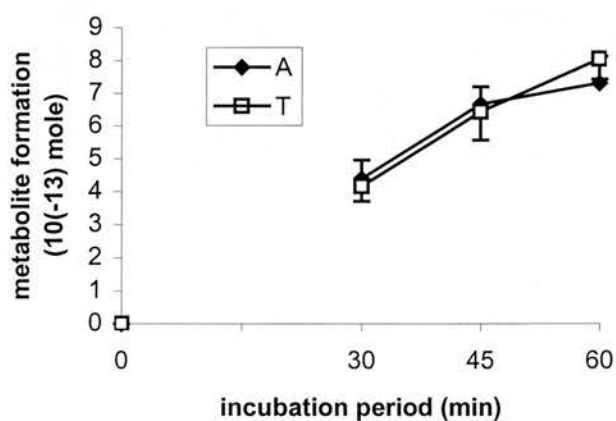
Using the same physiological substrate concentration, i.e. 10 nM, more samples of BPH tissue homogenates were assayed to compare 5 $\alpha$ -reduction of androstenedione and testosterone by the two isozymes (fig. 3.2). Assay of 5 $\alpha$ R1 (pH 7.5) in prostate tissue homogenates demonstrated approximately 50% more 5 $\alpha$ -reduction of androstenedione than testosterone (mean  $\pm$  s.d.;  $2.70 \pm 0.63$  and  $1.77 \pm 0.51$  pmol/mg protein/h respectively; fig. 3.2). In contrast, 5 $\alpha$ -reduction of androstenedione by 5 $\alpha$ R2 (pH 5.0) was similar to that of testosterone (mean  $\pm$  s.d.;  $4.70 \pm 0.95$  and  $4.86 \pm 0.97$  pmol/mg protein/h respectively; fig. 3.2).

Less than 10% of substrate was metabolised in each assay. Table 3.1 shows the profiles of steroid metabolites in 5 $\alpha$ -reductase assays. With androstenedione as substrate for 5 $\alpha$ R1 assays, the two most abundant metabolites, 5 $\alpha$ A and testosterone, accounted for 93.1% and 3.1% of all metabolites respectively. With testosterone as substrate, the major metabolite was DHT (94.7%) and androstenedione accounted for 3.4% of all metabolites measured (table 3.1). With both substrates, the profiles of metabolites obtained from 5 $\alpha$ R2 assays were very similar to those from 5 $\alpha$ R1 assays.

(a) pH 7.5

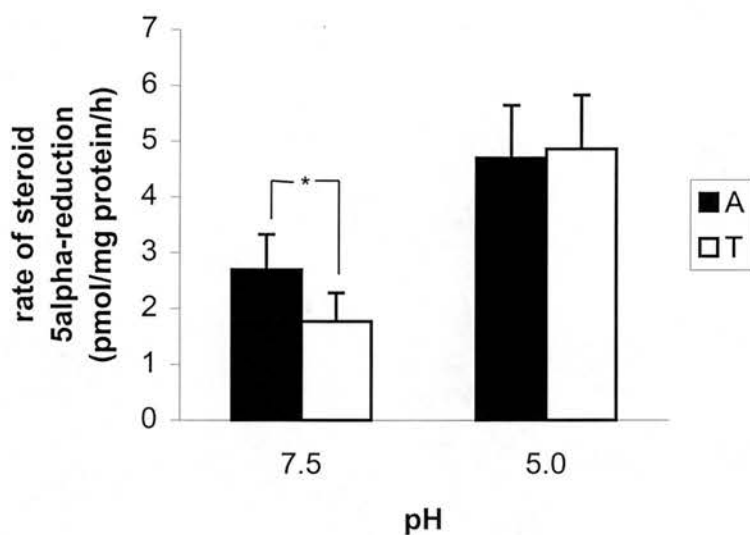


(b) pH 5.0



**Fig. 3.1 Time course of 5 $\alpha$ -reductase assay.** Duplicates of BPH tissue homogenates (prepared from two patients) containing 0.2 mg protein each were incubated with 10 pmol (10 nM in a final volume of 1 ml) radiolabelled androstenedione (A) or testosterone (T) for 0-60 min at (a) pH 7.5 for 5 $\alpha$ R1 isozyme or (b) pH 5.0 for 5 $\alpha$ R2 isozyme. Steroids were extracted and separated by thin layer chromatography as described in sections 2.3.2 & 2.7.1. Results are expressed as mean of duplicates  $\pm$  range.





**Fig 3.2 Comparison of androstenedione and testosterone 5 $\alpha$ -reduction at pH 7.5 and pH 5.0 in BPH tissue homogenates.** Tissue homogenates in duplicate containing 0.2 mg protein each were incubated for 30 min with 10 pmol (10 nM in a final volume of 1 ml) of androstenedione (A) or testosterone (T). Steroids were extracted and separated by thin layer chromatography as described in sections 2.3.2 & 2.7.1. Results are expressed as mean  $\pm$  s.d.  $n = 7$  patients (pH 7.5) and  $n = 5$  patients (pH 5.0). \* $P < 0.01$ .

5 $\alpha$ - reductase isozyme	substrate	percentage of metabolite (%)					
		A	5 $\alpha$ A	And/EA	T	DHT	Diols
type 1 (pH 7.5)	A	NA	93.1 $\pm$ 2.7	0.5 $\pm$ 0.3	3.1 $\pm$ 1.5	2.0 $\pm$ 0.8	1.3 $\pm$ 0.7
	T	3.4 $\pm$ 1.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	NA	94.7 $\pm$ 1.2	1.5 $\pm$ 0.9
type 2 (pH 5.0)	A	NA	94.0 $\pm$ 1.2	1.2 $\pm$ 0.5	2.7 $\pm$ 0.8	1.3 $\pm$ 0.3	0.8 $\pm$ 0.4
	T	1.9 $\pm$ 0.5	1.1 $\pm$ 0.4	0.5 $\pm$ 0.4	NA	95.8 $\pm$ 0.7	2.8 $\pm$ 0.3

Table 3.1 Profiles of metabolites formed from androstenedione and testosterone by 5 $\alpha$ -reductase isozymes. Tissue homogenates in duplicate containing 0.2 mg protein each were incubated for 30 min with 10 pmol (10 nM in a final volume of 1 ml) of androstenedione (A) or testosterone (T). Results are expressed as percentage of total amount of all metabolites (mean  $\pm$  sd). n = 4 patients. Steroids were extracted and separated by thin layer chromatography as described in sections 2.3.2 & 2.7.1. 5 $\alpha$ A, 5 $\alpha$ -androstanedione; And/EA, androsterone and epiandrosterone; DHT, 5 $\alpha$ -dihydrotestosterone; Diols, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol and 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol; NA, not applicable.

### 3.2 Establishment of a Co-transfection Model

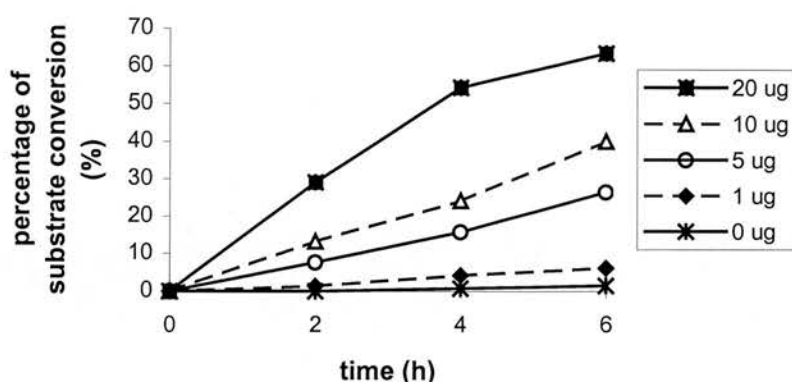
Results from the above 5 $\alpha$ -reductase assays demonstrate a higher conversion rate of androstenedione than testosterone by 5 $\alpha$ R1 in prostate tissue homogenates. Moreover, previous kinetic studies have also shown that aromatase exhibits higher affinity for androstenedione than testosterone (section 1.4.1). The hypothesis that 5 $\alpha$ R1 plays a role in the regulation of aromatase activity by limiting substrate availability to the latter was therefore tested using androstenedione as the androgen substrate. Because of the mixture of various steroid-metabolising enzymes expressed in the human prostate, cells co-transfected with plasmids encoding the two enzymes

5 $\alpha$ R1 and aromatase were first employed to investigate any possible interrelationship between the two enzymes.

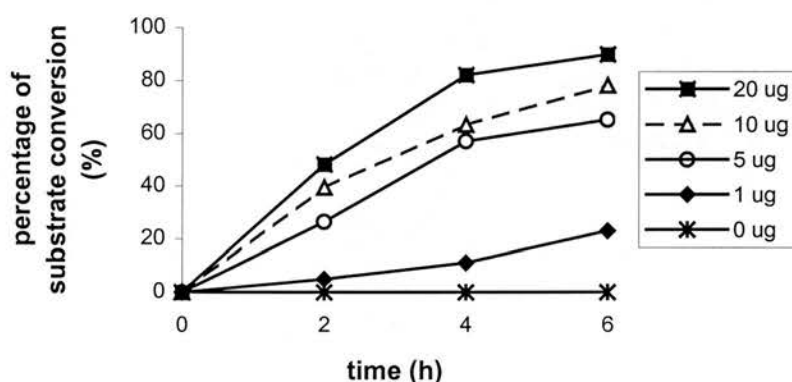
To set up a co-transfection model, three cell lines were screened for endogenous androgen-metabolising enzyme activities (table 3.2). No aromatase activity was detectable in any of the cell lines screened. Although C6 cells have been reported to express 5 $\alpha$ R1 activity (Morita et al. 1999), they were used in initial transfection experiments because they had the lowest levels of endogenous 5 $\alpha$ -reductase activity among the three cell lines. However, after transfection with an aromatase-encoding plasmid pCMV2.arom, C6 cells not only metabolised androstenedione to oestrone (aromatase activity) but also actively converted oestrone to oestradiol (17 $\beta$ HSD type 1-like activity; data not shown). They were therefore not used in subsequent experiments. Similarly, CHO cells were not used in transfection experiments because of their high 17 $\beta$ HSD type 3-like activity, converting androstenedione to testosterone. In comparison, COS-1 cells did not metabolise androstenedione to testosterone (17 $\beta$ HSD type 3-like activity) and were therefore employed to establish a co-transfection model. To reduce background 5 $\alpha$ -reductase activity in COS-1 cells, plating density was decreased to  $2 \times 10^5$  cells per 60 mm dish.

To establish a co-transfection model expressing both human 5 $\alpha$ R1 and aromatase, a series of preliminary experiments were carried out to determine the amount of each plasmid used in the co-transfection model and the duration of substrate incubation. COS-1 cells transfected with various amount of plasmid were incubated with 10 nM androstenedione; the medium was then collected after 2, 4 or 6 h (fig. 3.3). Figure 3.3a shows that in COS-1 cells transfected with pCMV2.5 $\alpha$ R1 only, 5 $\alpha$ -androstenedione (5 $\alpha$ A) formation within 4 h of incubation was essentially proportional to the amount of plasmid used (up to 20  $\mu$ g). In contrast, within 4 h of incubation, oestrone (E<sub>1</sub>) formation was proportionally increased in cells transfected with up to about 5  $\mu$ g of pCMV2.arom only (fig. 3.3b).

(a)  $5\alpha$ -androstanedione formation in cells transfected with pCMV2.5 $\alpha$ R1



(b) Oestrone formation in cells transfected with pCMV2.arom

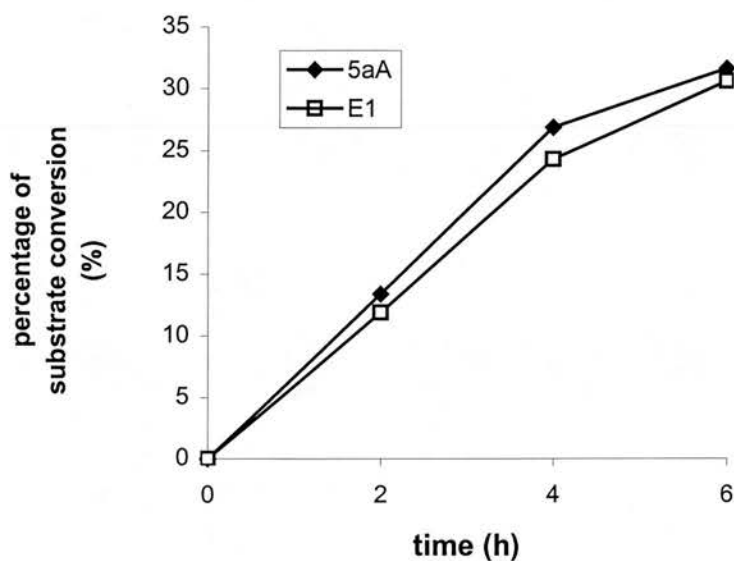


**Fig 3.3 Conversion of androstenedione to (a)  $5\alpha$ -androstanedione and (b) oestrone in COS-1 cells transfected with 1-20  $\mu$ g of either (a) pCMV2.5 $\alpha$ R1 or (b) pCMV2.arom.**  $2 \times 10^5$  COS-1 cells were plated in duplicate in 60 mm dishes on day 1 of the experiment. The expression plasmids were transfected into cells on day 2. Transfected cells were then incubated with 30 pmol (10 nM in a final volume of 3 ml) of androstenedione for 2, 4 or 6 h on day 3 of the experiment. Steroids were extracted and analysed by HPLC as described in sections 2.6.1 & 2.7.2. Results are expressed as mean of duplicates.

			Percentage of steroids					
Cell line	Substrate	Incubation	T	A	DHT	5 $\alpha$ A	androsterone	unknown
CHO	A	4 h	8%	86%	ND	6%	ND	ND
		24 h	49%	14%	6%	26%	ND	5%
	T	4 h	98%	ND	2%	ND	ND	ND
		24 h	93%	ND	7%	ND	ND	ND
COS-1	A	6 h	ND	98%	ND	2%	ND	ND
		24 h	ND	60%	ND	30%	7%	3%
	T	6 h	8%	90%	ND	2%	ND	ND
		24 h	ND	60%	ND	30%	6%	4%
C6	A	4 h	ND	100%	ND	ND	ND	ND
		24 h	3%	94%	ND	3%	ND	ND
	T	4 h	100%	ND	ND	ND	ND	ND
		24 h	96%	ND	4%	ND	ND	ND

Table 3.2 Endogenous androgen-metabolising activities in cell lines.  $3 \times 10^5$  cells per 60 mm dish were plated in DMEM supplemented with 10% DCC-FCS 48 h before incubation with 30 pmol radiolabelled substrate (10 nM in a final volume of 3 ml). Medium was collected after 4, 6 or 24 h incubation and steroids were analysed by HPLC. T, testosterone; A, androstenedione; DHT, dihydrotestosterone; 5 $\alpha$ A, 5 $\alpha$ -androstenedione; ND, not detectable. No oestrone or oestradiol was detectable within 24 h incubation.

In subsequent experiments to set up a co-transfection model, combinations of various amounts of pCMV2.5 $\alpha$ R1 and pCMV2.arom as well as 2  $\mu$ g pSV2L (luciferase-encoding plasmid as internal control) were tested. Figure 3.4 shows that in COS-1 cells co-transfected with 10  $\mu$ g pCMV2.5 $\alpha$ R1, 3  $\mu$ g pCMV2.arom and 2  $\mu$ g pSV2L, formation of both 5 $\alpha$ A and E<sub>1</sub> was proportional with time up to 4 h of incubation. Endogenous metabolism of androstenedione to 5 $\alpha$ A in COS-1 cells mock-transfected with 13  $\mu$ g pCMV2 and 2  $\mu$ g pSV2L was approximately 2% in 6 h. There was no background aromatase activity. No other steroid metabolites were detectable within 6 h of incubation. In all subsequent experiments using this co-transfection model, COS-1 cells were therefore incubated for 3 h.



**Fig 3.4 Metabolism of androstenedione in co-transfection model.** COS-1 cells were co-transfected with 10  $\mu$ g pCMV2.5 $\alpha$ R1, 3  $\mu$ g pCMV2.arom and 2  $\mu$ g pSV2L. Apart from 5 $\alpha$ -androstenedione (5 $\alpha$ A) and oestrone (E1), no other steroid metabolites were detectable within 6 h incubation. Experimental procedure was the same as that described in fig. 3.3. Results are expressed as mean of duplicates and corrected by baseline conversion of androstenedione to 5 $\alpha$ A in mock-transfected COS-1 cells.

### 3.3 Interaction between 5 $\alpha$ R1 and Aromatase in a Co-transfection Model

To investigate any interaction between 5 $\alpha$ R1 and aromatase,  $2 \times 10^5$  COS-1 cells were plated in triplicate in DMEM supplemented with 10% DCC-FCS on day 1 of the experiment. COS-1 cells were then co-transfected with a total of 15  $\mu$ g plasmid DNA (10  $\mu$ g pCMV2.5 $\alpha$ R1, 3  $\mu$ g pCMV2.arom and 2  $\mu$ g pSV2L) on day 2. On day 3 of the experiment, transfected cells were incubated with 30 pmol (10 nM in a final volume of 3 ml medium) of androstenedione for 3 h to achieve similar levels of 5 $\alpha$ A and E<sub>1</sub> formation (approximately 20% each). Ethanol or one of the following 2 drugs was added onto the monolayer cell cultures at the time of substrate incubation:

- a. 1-10 nM aromatase-specific inhibitor, letrozole (Bhatnagar et al. 1990; Novartis, New Jersey, USA; a gift of Dr. Colin Bayne, University of Edinburgh);
- b. 1-10 nM 5 $\alpha$ R1-specific inhibitor, LY306089 (Kaefer et al. 1996; Eli Lilly Laboratories, Greenfield, Indiana, USA).

At the end of the incubation period, steroids were extracted from the medium and analysed by HPLC; cells were lysed and luciferase activity was determined as described in sections 2.5.3 & 2.5.4.

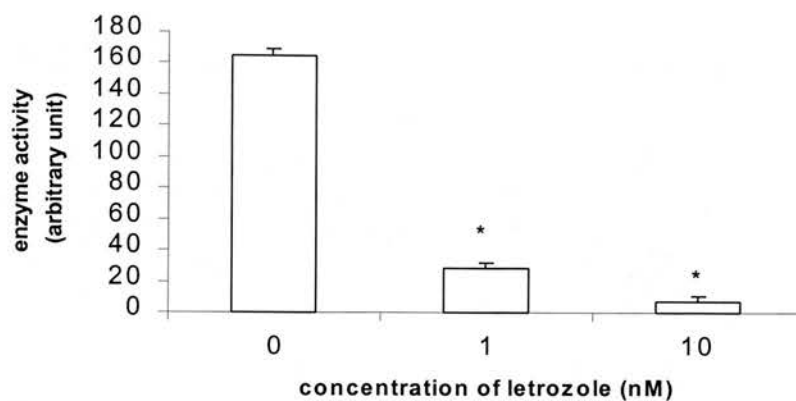
#### 3.3.1 Effects of letrozole

In COS-1 cells transfected with pCMV2.arom alone, addition of the aromatase inhibitor letrozole inhibited androstenedione aromatisation in a dose-dependent manner as expected (fig. 3.5a). 5 $\alpha$ R1 activity encoded by pCMV2.5 $\alpha$ R1 was not affected by letrozole (fig. 3.5b). In cells co-transfected with both pCMV2.arom and pCMV2.5 $\alpha$ R1, letrozole inhibited aromatase activity in a dose-dependant manner and did not affect the conversion of androstenedione to 5 $\alpha$ A (fig. 3.5c).

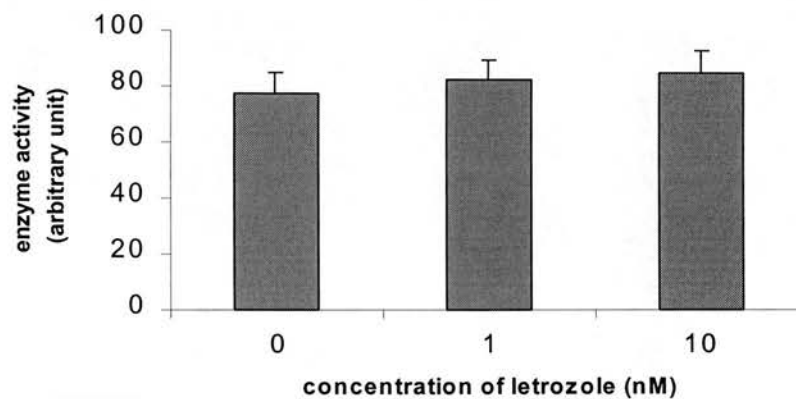
**Fig. 3.5 Effects of letrozole (aromatase inhibitor) on enzyme activities in COS-1 cells transfected with (a) pCMV2.arom (aromatase-encoding plasmid) only, (b) pCMV2.5 $\alpha$ R1 (5 $\alpha$ R1-encoding plasmid) only, and (c) both pCMV2.arom and pCMV2.5 $\alpha$ R1.** Enzyme activities are expressed as mean of triplicates  $\pm$  s.e.m. Arbitrary unit of steroid-metabolising enzyme activity is calculated by the following formula: steroid formation (pmol)  $\times 10^6$  / luciferase activity (relative light unit). Results are representative of 3 different experiments. \*P<0.01 compared with controls. Open bars, aromatase; solid bars, 5 $\alpha$ R1.



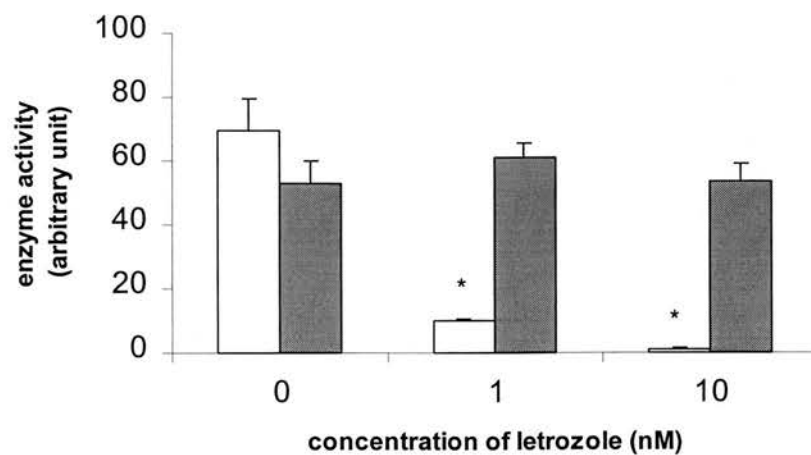
**(a) Aromatase only**



**(b) 5 $\alpha$ R1 only**



**(c) Both aromatase & 5 $\alpha$ R1**



### 3.3.2 Effects of LY306089

In contrast, the 5 $\alpha$ R1 inhibitor LY306089 (10 nM) had no effect on aromatase activity in cells transfected with pCMV2.arom alone (fig. 3.6a) but inhibited 5 $\alpha$ -reduction in cells transfected with pCMV2.5 $\alpha$ R1 (fig. 3.6b). In cells co-transfected with both pCMV2.arom and pCMV2.5 $\alpha$ R1, addition of 10 nM LY306089 decreased 5 $\alpha$ R1 activity by 64% and led to a concomitant 45% increase in aromatase activity (fig. 3.6c).

Collectively, these data indicate that in COS-1 cells co-expressing human 5 $\alpha$ R1 and aromatase, the former enzyme regulates the activity of the latter but not vice versa.

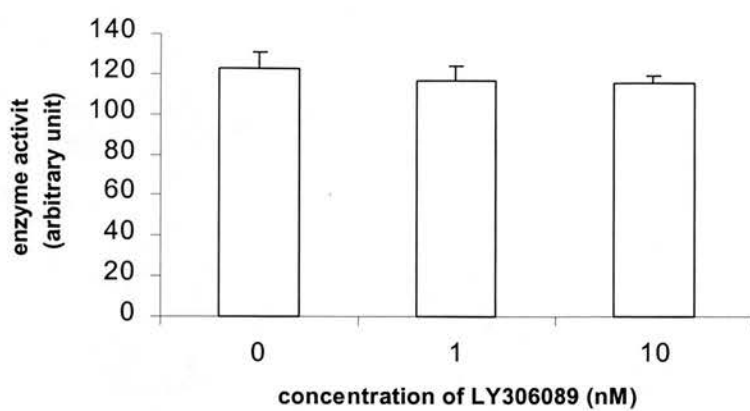
## 3.4 Aromatase mRNA Expression in Prostatic Cells in Primary Culture

Aromatase expression in the prostate is controversial (reviewed in section 1.4.3). Therefore, before investigating whether the interaction between 5 $\alpha$ R1 and aromatase demonstrated in the above co-transfection model also occurs in prostatic cells, the expression of aromatase activity and mRNA in primary cultures of prostatic cells was examined using aromatase enzyme assays and RT-PCR respectively.

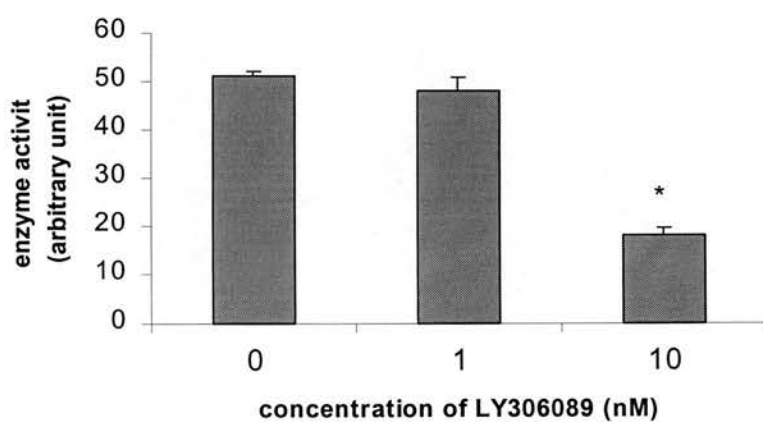
To determine if prostatic cells in primary culture express aromatase mRNA, total RNA was prepared from epithelial cells and fibroblasts in primary culture derived from 3 BPH patients and analysed for aromatase mRNA by RT-PCR. All RT reactions were carried out under identical conditions at the same time. Also, to determine whether aromatase mRNA levels are affected by the process of cell passaging, serial dilutions of fibroblast (passage numbers 1-5) RT reactions were used in the PCR reactions. Although the PCR used was not quantitative, the results obtained from serial dilutions of RT reactions give an indication as to the relative levels of aromatase mRNA expression in fibroblasts at different passage numbers. Only epithelial cells of passage number 1 were used because they did not grow well after the second passage.

**Fig. 3.6 Effects of LY306089 (5 $\alpha$ R1 inhibitor) on enzyme activities in COS-1 cells transfected with (a) pCMV2.arom (aromatase-encoding plasmid) only, (b) pCMV2.5 $\alpha$ R1 (5 $\alpha$ R1-encoding plasmid) only, and (c) both pCMV2.arom and pCMV2.5 $\alpha$ R1.** Enzyme activities are expressed as mean of triplicates  $\pm$  s.e.m. Arbitrary unit of steroid-metabolising enzyme activity is calculated by the following formula: steroid formation (pmol)  $\times 10^6$  / luciferase activity (relative light unit). Results are representative of 3 different experiments. \*P<0.01 compared with controls. Open bars, aromatase; solid bars, 5 $\alpha$ R1.

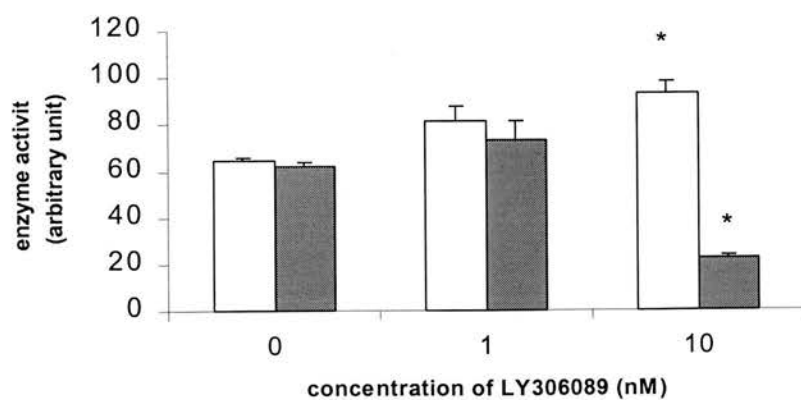
**(a) Aromatase only**



**(b) 5 $\alpha$ R1 only**



**(c) Both aromatase & 5 $\alpha$ R1**



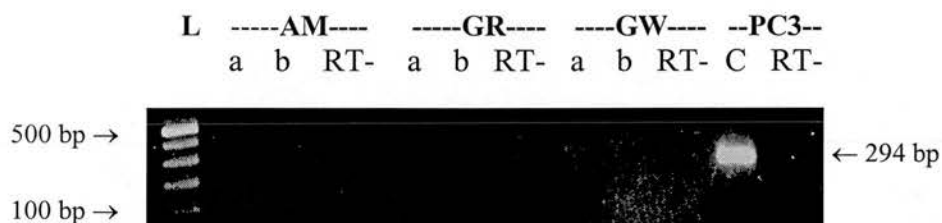
#### 3.4.1 Epithelial cells

None of the three epithelial cell samples expressed aromatase mRNA (fig. 3.7 and table 3.3). Positive control reactions using RNA prepared from PC3 cells confirmed that the PCR reaction yielded a product of 294 bp (fig. 3.7). Identity of the 294-bp PCR product was verified by the restriction enzyme *AvaII* (fig. 3.8). Control PCR reactions using primers for the housekeeping GAPDH gene to detect GAPDH cDNA in the above epithelial cell RT reactions demonstrated that the RNA samples were not degraded and reverse transcription had taken place (data not shown).

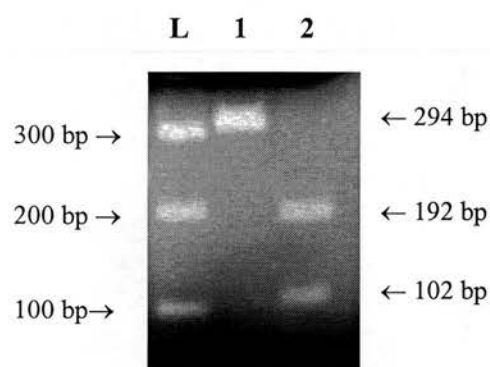
#### 3.4.2 Fibroblasts

In contrast to epithelial cells, most samples of fibroblasts (up to passage number 5) expressed aromatase mRNA (fig. 3.9 & table 3.3). RT-PCR products were, however, only detectable using undiluted RT reactions, indicating that the levels of aromatase mRNA expression in fibroblasts were relatively low. In comparison, using fibroblast RT reactions diluted up to  $10^4$ -fold, control PCR reactions detected the PCR product of GAPDH in all samples, confirming that the RNA/cDNA samples were not degraded (data not shown).

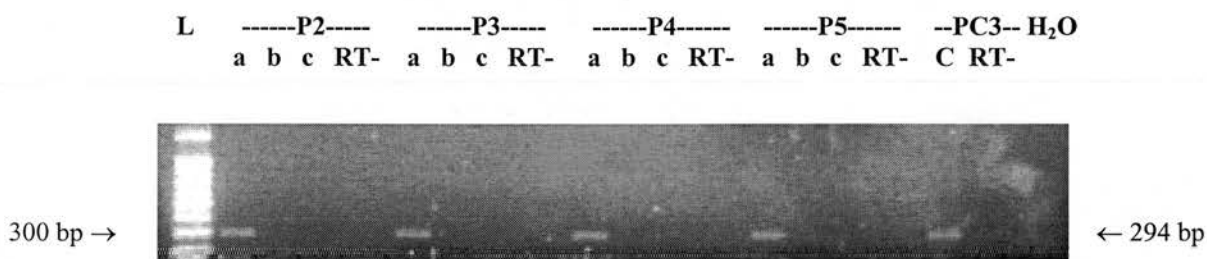
Taken together, these results indicate that aromatase mRNA is expressed in prostatic fibroblasts in primary culture but not in epithelial cells. With increasing passage number, no consistent change in aromatase mRNA levels in fibroblasts is obvious.



**Fig. 3.7 Aromatase mRNA is not expressed in epithelial cells cultured from BPH tissues.** Total RNA was prepared from epithelial cells (passage number 1) of 3 different BPH patients (AM, GR, GW) and from PC3 cells. The same amount of RNA from each sample underwent RT at the same time under identical conditions. PCR reactions (40 cycles) were carried out on (a) undiluted, (b) 10-fold diluted or 100-fold diluted (not shown) RT reactions. RT-, negative control in which reverse transcriptase was omitted; C, positive control using RNA isolated from PC3 cells. L, 100-bp DNA ladder.



**Fig. 3.8 Restriction enzyme digestion of aromatase PCR product.** The 294-bp aromatase PCR product was digested with *Ava*II to confirm its identity. *Ava*II produced two fragments of 102 and 192 bp. L, 100-bp DNA ladder; lane 1, 294-bp undigested PCR product; lane 2, *Ava*II digestion of PCR product.



**Fig. 3.9 Aromatase mRNA is expressed in fibroblasts cultured from BPH tissue.**

Total RNA was prepared from fibroblasts (passage numbers 2 to 5) of BPH patient GR and from PC3 cells. The same amount of RNA from each sample underwent RT at the same time under identical conditions. PCR reactions (40 cycles) were carried out on (a) undiluted, (b) 10-fold diluted, and (c) 100-fold diluted RT reactions. L, 100-bp DNA ladder; P2-P5, fibroblasts of passage numbers 2 to 5; RT-, negative control in which reverse transcriptase was omitted; C, positive control using RNA isolated from PC3 cells; H<sub>2</sub>O, negative PCR control with water in place of RT reaction. RT-PCR reactions carried out on total RNA prepared from two other patients' fibroblasts generated similar results (table 3.3).



		AM			GR			GW		
		cDNA dilution			cDNA dilution			cDNA dilution		
cell type	passage number	1:1	1:10	1:100	1:1	1:10	1:100	1:1	1:10	1:100
<b>Ep</b>	P1	-	-	-	-	-	-	-	-	-
<b>Fb</b>	P1	-	-	-	nt	nt	nt	+	-	-
	P2	+	-	-	+	-	-	+	-	-
	P3	+	-	-	+	-	-	+	-	-
	P4	+	-	-	+	-	-	+	-	-
	P5	+	-	-	+	-	-	+	-	-

Table 3.3 Aromatase mRNA is expressed in fibroblasts but not epithelial cells in primary culture. Total RNA was prepared from epithelial cells (Ep, passage number 1) and fibroblasts (Fb, passage number up to 5) derived from three BPH tissue samples (designated AM, GR and GW). PCR reactions (40 cycles) were carried out using RT reactions diluted up to 100-fold. +, present; -, absent; nt, not tested. The PCR was repeated using the undiluted fibroblast RT reaction (passage number 1, patient AM) and again yielded no PCR product.

### 3.5 Androstenedione Metabolism in Prostatic Cells in Primary Culture

Previous studies from the Prostate Research Group (University of Edinburgh) have shown that BPH-derived fibroblasts and epithelial cells in primary culture express 5 $\alpha$ R1 but not 5 $\alpha$ R2 activity (Bayne et al. 1998). Results in this chapter have already confirmed the expression of aromatase mRNA in prostatic fibroblasts. The next set of experiments aimed at investigating any aromatase enzyme activity in prostatic cells in primary culture with a view to ascertain whether an interaction between 5 $\alpha$ R1 and aromatase also exists in prostatic cells.

### 3.5.1 Fibroblasts

Preliminary experiments showed that in fibroblasts, the conversion of androstenedione to  $5\alpha\text{A}$  (by  $5\alpha\text{R1}$ ) and  $\text{E}_1$  (by aromatase) was essentially proportional to the duration of incubation up to 52 h (figs. 3.10a & 3.10b). In comparison, testosterone formation (by  $17\beta\text{HSD}$ ) increased with time only within 26 h (fig. 3.10c). The activity of  $5\alpha\text{R1}$  was the highest among the three enzymes. In subsequent experiments to examine the relationship among these enzymes, fibroblasts were incubated with androstenedione for 24 h to allow formation of reasonable amount of all 3 metabolites.

### 3.5.2 Epithelial cells

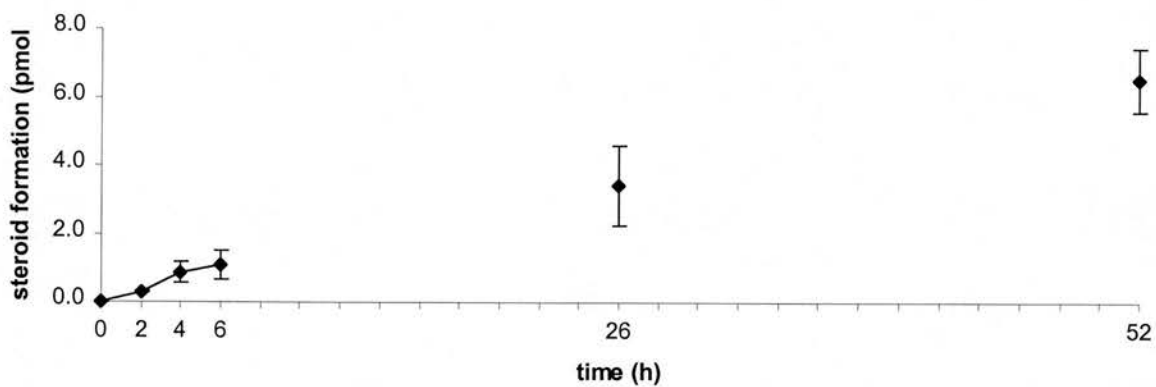
In comparison, the conversion of androstenedione in epithelial cells to both  $5\alpha\text{A}$  and testosterone was more or less proportional to the duration of incubation up to 6 h, with the rate of  $5\alpha\text{A}$  formation higher than that of testosterone (fig. 3.11). However, no  $\text{E}_1$  or  $\text{E}_2$  was detectable within 24 h. Epithelial cells were incubated with androstenedione for 4 h in subsequent experiments to study the effects of enzyme inhibitors.

## 3.6 Interaction between $5\alpha\text{R1}$ and Aromatase in Prostatic Cells in Primary Culture

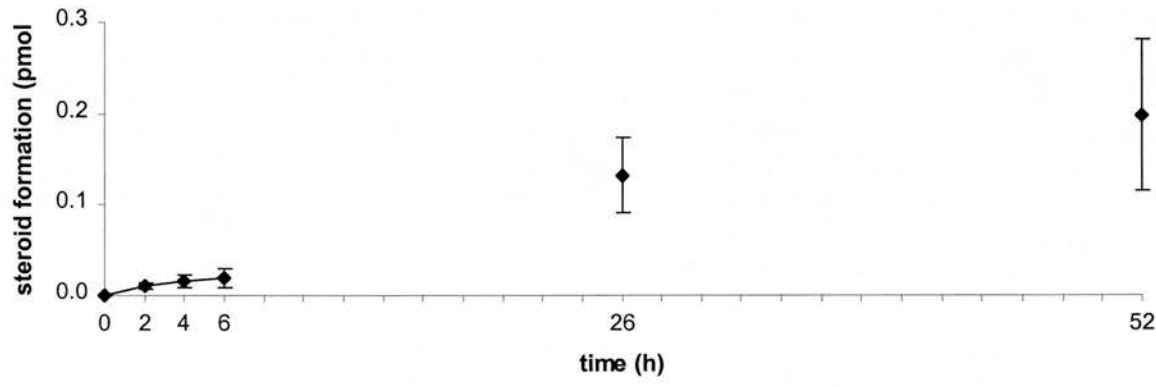
Results from COS-1 cells co-transfected with pCMV2.arom and pCMV2. $5\alpha\text{R1}$  suggest that  $5\alpha\text{R1}$  inhibition leads to an increase in aromatase activity by alleviating  $5\alpha\text{R1}$  competition for the common substrate androstenedione. To ascertain if the regulatory interaction between aromatase and  $5\alpha\text{R1}$  observed in co-transfected cells also occurs in a more physiologically relevant system, the metabolism of androstenedione in prostatic fibroblasts and epithelial cells under the influence of LY306089 ( $5\alpha\text{R1}$ -specific inhibitor) and letrozole (aromatase-specific inhibitor) was investigated.

**Fig. 3.10 Time course of androstenedione metabolism in fibroblasts in primary culture.**  $3 \times 10^5$  fibroblasts/well in phenol red-free RPMI supplemented with 10% DCC-FCS were seeded in duplicate into 6-well plates 48 h before incubation with 40 pmol (20 nM in a final volume of 2 ml, approximately  $8 \times 10^6$  dpm) androstenedione. Medium was collected 0, 2, 4, 6, 26 or 52 h later. Steroids were separated by thin-layer chromatography and the formation of (a)  $5\alpha$ A, (b)  $E_1$  and (c) testosterone was quantitated by scintillation counting. Results are expressed as mean  $\pm$  s.d. (n=4) from 2 separate experiments using fibroblasts derived from 2 BPH patients.

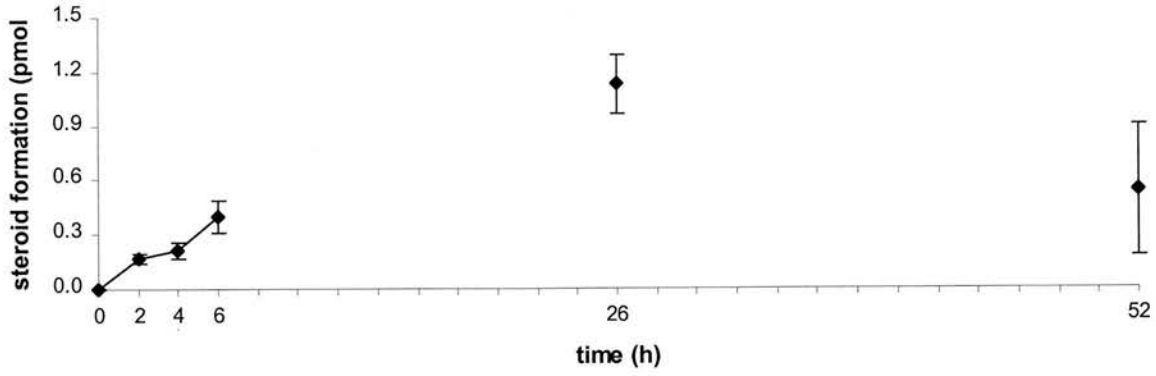
(a) 5 $\alpha$ A formation



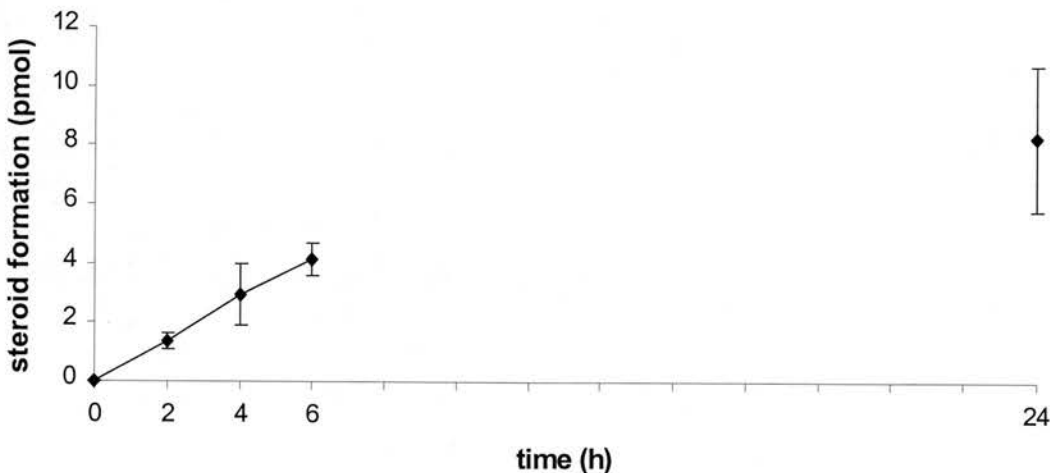
(b) E<sub>1</sub> formation



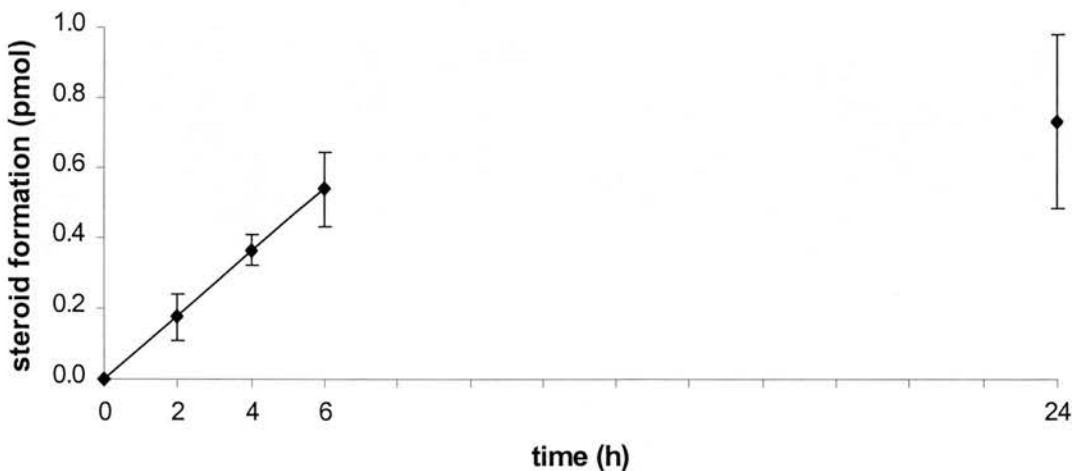
(c) testosterone formation



(a) 5 $\alpha$ A formation



(b) testosterone formation



**Fig. 3.11 Time course of androstenedione metabolism in epithelial cells in primary culture.**  $2 \times 10^5$  cells/well in serum-free EGM were seeded in duplicate into 6-well plates 48 h before incubation with 40 pmol (20 nM in a final volume of 2 ml, approximately  $8 \times 10^6$  dpm) androstenedione. Medium was collected 0, 2, 4, 6 or 24 h later. Steroids were separated by thin-layer chromatography and the formation of (a) 5 $\alpha$ A and (b) testosterone was quantitated by scintillation counting. No oestrone formation was detectable up to 24 h. Results are expressed as mean  $\pm$  s.d. ( $n=4$ ) from 2 separate experiments using epithelial cells derived from 2 BPH patients.

### 3.6.1 Fibroblasts

In prostatic fibroblasts, androstenedione was metabolised to 5 $\alpha$ A (by 5 $\alpha$ R1, 331  $\pm$  29 fmol/mg protein/h), testosterone (17 $\beta$ HSD type 5-like activity, 101  $\pm$  18 fmol/mg protein/h) and E<sub>1</sub> (by aromatase, 13  $\pm$  1 fmol/mg protein/h) (fig. 3.12).

5 $\alpha$ A formation was inhibited by the 5 $\alpha$ R1 inhibitor LY306089 in a dose-dependent manner but not affected by the aromatase inhibitor letrozole (fig. 3.12a). Testosterone formation was unaffected by either of the two drugs (fig. 3.12b). Figure 3.12c shows that letrozole inhibited E<sub>1</sub> formation as expected in a dose-dependent manner whereas addition of LY306089 (100 nM) led to a significant increase in aromatase activity in fibroblasts. These data indicate that by inhibiting 5 $\alpha$ R1 activity, LY306089 can increase aromatase activity not only in the above co-transfection model but also in fibroblasts in primary culture.

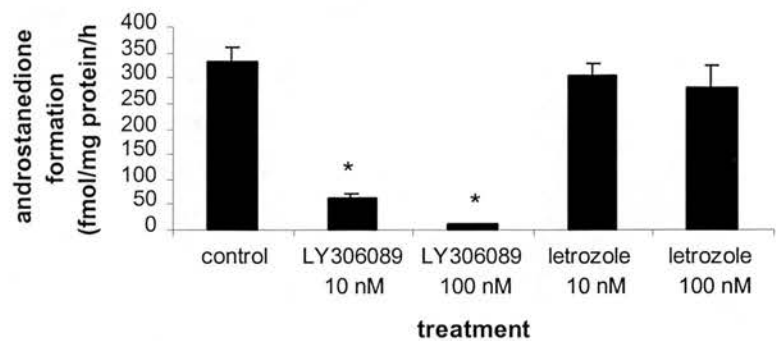
### 3.6.2 Epithelial cells

In prostatic epithelial cells, androstenedione was metabolised to 5 $\alpha$ A (by 5 $\alpha$ R1, 1156  $\pm$  103 fmol/mg protein/h) and to testosterone (17 $\beta$ HSD type 5-like activity, 153  $\pm$  39 fmol/mg protein/h) (fig. 3.13). Unlike fibroblasts, no aromatisation of androstenedione to E<sub>1</sub> was detectable. It was thus impossible to confirm the regulatory interaction between 5 $\alpha$ R1 and aromatase, which was observed in the above co-transfection model and in fibroblasts.

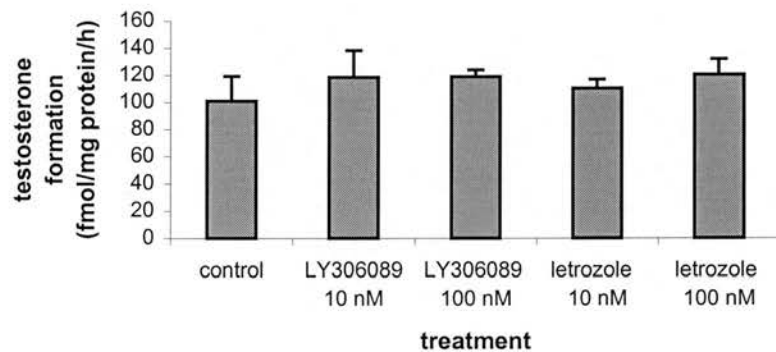
In epithelial cells, LY306089 inhibited 5 $\alpha$ A formation as expected in a dose-dependent manner (fig. 3.13a) but did not affect the reduction of androstenedione to testosterone (fig. 3.13b) or produce any aromatase activity (data not shown).

**Fig. 3.12 Effects of LY306089 and letrozole on androstenedione metabolism in fibroblasts in primary culture.**  $3 \times 10^5$  fibroblasts/well in phenol red-free RPMI supplemented with 10% DCC-FCS were seeded in triplicate into 6-well plates. 48 h later, 2 ml of the same medium containing 20 nM androstenedione and either LY306089 or letrozole was added to the cells. Medium was collected after 24 h incubation. Fibroblasts were lysed and protein concentrations determined as described in sections 2.6.1 & 2.3.4 respectively. Steroids were then extracted from culture medium and separated by thin layer chromatography. The formation of (a)  $5\alpha A$ , (b) testosterone and (c)  $E_1$  was quantitated by scintillation counting. Values are mean of triplicates  $\pm$  s.e.m. \* $P < 0.01$ ,  $^+P < 0.05$  compared with controls. Results are representative of 3 different experiments using fibroblasts from 3 BPH patients.

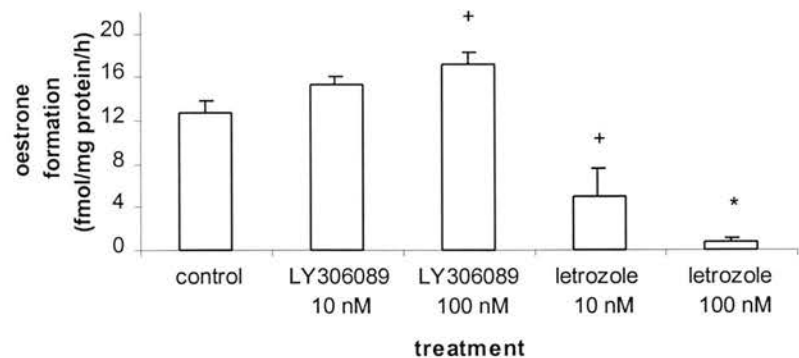
(a) 5αR1 activity



(b) 17βHSD activity



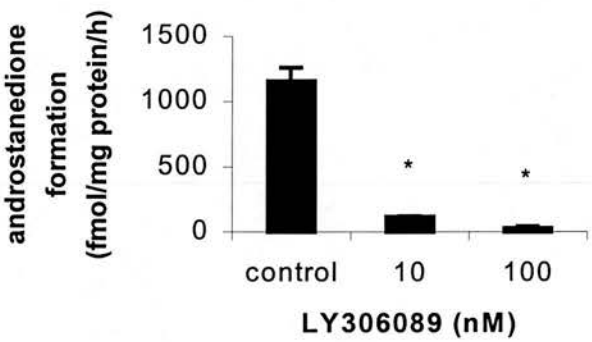
(c) aromatase activity



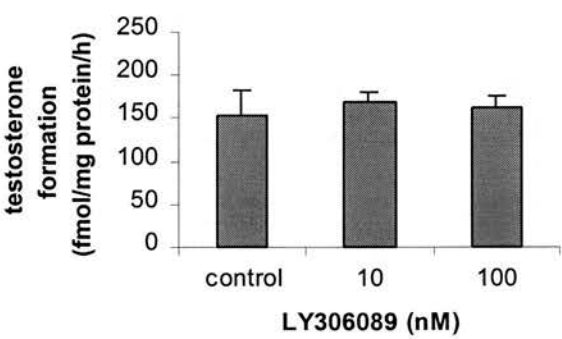


**Fig. 3.13 Effects of LY306089 on androstenedione metabolism in epithelial cells in primary culture.**  $2 \times 10^5$  cells/well in serum-free EGM were seeded in triplicate into 6-well plates. 48 h later, 2 ml of the same medium containing 20 nM androstenedione and 10-100 nM LY306089 was added to the cells. Medium was collected after 4 h incubation. Epithelial cells were lysed and protein concentrations determined as described in sections 2.6.1 & 2.3.4 respectively. Steroids were then extracted from culture medium and separated by thin layer chromatography. The formation of (a)  $5\alpha A$ , and (b) testosterone was quantitated by scintillation counting. No aromatase activity (formation of  $E_1$  or  $E_2$ ) was detectable in epithelial cells. Values are mean of triplicates  $\pm$  s.e.m. \* $P < 0.01$  compared with controls. Results are representative of 3 different experiments using epithelial cells from 3 BPH patients.

**(a) 5 $\alpha$ R1 activity**



**(b) 17 $\beta$ HSD activity**



### 3.7 Discussion

Despite recent reports of 5 $\alpha$ R1 mRNA (Habib et al. 1998, Ihle et al. 1999), protein (Bonkhoff et al. 1996, Negri-Cesi et al. 1998) and enzyme activity (Bayne et al. 1998) in the human prostate, its functions in this gland are unclear. In tissues such as the prostate which express both 5 $\alpha$ R1 and aromatase, one possible role for 5 $\alpha$ R1 is the regulation of aromatase activity, which shares common substrates with 5 $\alpha$ R1. To test this hypothesis, the interaction between the two enzymes was investigated firstly in a co-transfection model and then in prostatic cells in primary culture. In COS-1 cells co-transfected with expression plasmids encoding human 5 $\alpha$ R1 and aromatase, inhibition of 5 $\alpha$ R1 increased aromatase activity but the converse was not true; inhibition of aromatase did not affect 5 $\alpha$ R1 activity. This pattern of regulatory interaction also occurred with the endogenous enzymes in prostatic fibroblasts in primary culture.

The above observations are in keeping with data from female mice homozygous for a targeted disruption of the 5 $\alpha$ R1 gene (Mahendroo et al. 1997). In pregnant mice, impairment of androgen 5 $\alpha$ -reduction to non-aromatisable forms due to 5 $\alpha$ R1 deficiency led to increased androgen aromatisation, elevation in plasma oestrogen levels and mid-gestational fetal loss. This observation of increased plasma oestrogen concentrations in 5 $\alpha$ R1-deficient mice indicates that a normal role for 5 $\alpha$ R1 is to prevent the metabolism of placental androgens to oestrogens by converting aromatisable androgens to non-aromatisable forms (Mahendroo et al. 1997). However, no specific role for 5 $\alpha$ R1 has yet been proposed in male mice or in man. Recent findings that male mice lacking 5 $\alpha$ R2 or both 5 $\alpha$ -reductase isozymes only had mild virilisation defects (Mahendroo et al. 2001) are in sharp contrast to human male pseudohermaphrodites characterised by rudimentary prostates and female or ambiguous external genitalia due to 5 $\alpha$ R2 deficiency (Andersson et al. 1991) (reviewed in section 1.3.2). To date, no 5 $\alpha$ R1 deficiency has been identified in humans. These data suggest that functions of the 5 $\alpha$ -reductase isozymes can vary

significantly from species to species.  $5\alpha$ R1 was shown to regulate aromatisation of androgens to oestrogens in the uteroplacental unit of the female mouse (Mahendroo et al. 1997); here for the first time  $5\alpha$ R1 was demonstrated to have the potential to play a similar role in the prostate of human males.

$5\alpha$ -reductase activities in BPH tissue homogenates in the present study (1.77-2.70 pmol/mg protein/h for  $5\alpha$ R1 and 4.70-4.86 pmol/mg protein/h for  $5\alpha$ R2) are comparable to the  $5\alpha$ -reductase activity of 6.4 pmol/mg protein/h in prostate cell suspensions (presumably a mixture of activities of both isozymes because of living cells) reported by Smith et al. (1996). In keeping with the general consensus that  $5\alpha$ R2 is the predominant isozyme in the human prostate, here it is shown that in BPH tissue homogenates  $5\alpha$ R2 activities were higher than those of  $5\alpha$ R1.

Although both  $5\alpha$ -reductase isozymes can convert androstenedione to  $5\alpha$ A and testosterone to DHT, in BPH tissue homogenates  $5\alpha$ R1 metabolised more androstenedione than testosterone at physiological substrate concentrations. These results are also consistent with previous kinetic data from cell lines expressing  $5\alpha$ R1 (table 1.2, p.16).  $5\alpha$ R1 expressed in CHO cells was 6-11 times more efficient at metabolising androstenedione than testosterone, whereas  $5\alpha$ R2 expressed in the same cell line demonstrated similar  $V_{\max}/K_m$  with both substrates (Thigpen et al. 1993, Levy et al. 1995). By selectively metabolising androstenedione,  $5\alpha$ R1 in the prostate may therefore function as a catabolic enzyme in limiting the conversion of androstenedione to other active steroids including testosterone and oestrogens. A catabolic role for  $5\alpha$ R1 in limiting substrate availability to other enzymes is likely to be particularly important in the human prostate, in which the androstenedione/testosterone ratio is high compared to that in plasma (Habib et al. 1976) and androstenedione can be metabolised by aromatase or  $17\beta$ HSD to active steroids. Whilst in theory this regulatory function in androstenedione metabolism could also be served by  $5\alpha$ R2, evidence suggests that the primary role of  $5\alpha$ R2 is to convert testosterone to DHT (Imperato-McGinley et al. 1981, Andersson et al. 1991, Katz et al. 1995). Compared to normal males, individuals with homozygous  $5\alpha$ R2

deficiency have increased plasma testosterone/DHT ratios but normal oestrogen levels (Imperato-McGinley et al. 1981). Females homozygous for 5 $\alpha$ R2 deficiency are also characterised by elevated plasma testosterone/DHT ratios but have normal oestradiol levels throughout the menstrual cycle (Katz et al. 1995).

In both epithelial cells and fibroblasts in the present study, inhibition of 5 $\alpha$ R1 did not increase testosterone formation from androstenedione. This is most likely attributed to the strong oxidative 17 $\beta$ HSD type 2-like activity in these cells converting testosterone to androstenedione (Delos et al. 1995). Similarly, despite aromatase activity, oestradiol formation was not detectable in fibroblasts where androstenedione was converted to testosterone, a precursor of oestradiol. This is also possibly a result of high 17 $\beta$ HSD type 2-like activity present in fibroblasts, rapidly oxidising any oestradiol formed from testosterone aromatisation to oestrone. Interconversion between oestradiol and oestrone in prostatic cells in primary culture is a subject of the next chapter.

Both aromatase mRNA expression and enzyme activity in the present study were demonstrated in prostatic fibroblasts in primary culture but were undetectable in epithelial cells. These observations on the differential distribution of aromatase between the stromal and epithelial compartments are consistent with previous immunohistochemical studies (Matzkin & Soloway 1992, Hiramatsu et al. 1997), in which aromatase protein was localised mainly to the stroma of normal, BPH and carcinomatous prostate tissues.

The aromatase activities in prostatic fibroblasts in primary culture reported here ( $13 \pm 1$  fmol/mg protein/h) are similar to androstenedione aromatisation demonstrated in fibroblasts derived from BPH and prostatic cancer tissues (8-116 fmol/mg protein/h, Schweikert 1979). In comparison, oestrone formation from androstenedione ranged from 22 to 284 fmol/mg protein/h in homogenates prepared from normal and BPH tissues (Stone et al. 1986).

In summary, this chapter has shown that in human prostate, 5 $\alpha$ R1 has the potential to play a role in regulating aromatase activity, which is found in fibroblasts but not in epithelial cells in primary culture. In the next chapter, the expression of oestrogen receptors, which are essential for the action of oestrogens, in prostatic cells in primary culture and also the effects of oestrogens on these cells are investigated.

## **Chapter 4**

### **Results:**

#### ***Prostatic Cells are Oestrogen Target Cells and Capable of Oestrogen Catabolism***

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- 4.1 Oestrogen receptor mRNA expression in prostatic cells in primary culture
- 4.2 Growth response of prostatic cells in primary culture to oestrogens
- 4.3 Stimulatory effects of E<sub>2</sub> and 3 $\beta$ Adiol on prostatic fibroblasts in primary culture are antagonised by the anti-oestrogen ICI 182780
- 4.4 Oestrogen catabolism in prostatic cells in primary culture
- 4.5 Discussion

Clinical presentation of BPH manifests at an age when plasma ratios of oestrogens/androgens are increasing, implicating an imbalance between oestrogens and androgens as a factor in the pathogenesis of BPH. The previous chapter has also shown aromatase mRNA expression and activity in prostatic fibroblasts, demonstrating that prostatic cells have the potential to synthesise oestrogens from androgen precursors. Locally produced oestrogens may have intracrine/autocrine effects on fibroblasts themselves or act on neighbouring epithelial cells in a paracrine manner, provided that the appropriate receptors are expressed in these cells. To date, it is not entirely clear whether oestrogens, derived from plasma or locally produced, can cause or potentiate BPH, a process of excessive cellular proliferation of both epithelial and stromal components within the prostate gland.

At the beginning of this part of the study, it was hypothesised that prostatic cells are not only oestrogen target cells expressing oestrogen receptors, but are also capable of modulating the effects of oestrogens by active oestrogen metabolism. To test this hypothesis, various oestrogen response parameters were explored in prostatic cells in primary culture. Firstly, mRNA expression of both oestrogen receptor (ER) subtypes, crucial for receptor-mediated oestrogen action, in prostatic cells in primary culture was examined using RT-PCR. Secondly, effects of oestrogens on the proliferation of prostatic cells in primary culture were investigated, and the anti-oestrogen ICI 182780 was employed to ascertain if such effects are ER-mediated. Thirdly, metabolism of the natural oestrogens oestradiol and oestrone in prostatic cells has not been reported in the literature; experiments were therefore carried out to determine if prostatic cells in primary culture were capable of modulating oestrogen action by active oestrogen metabolism.



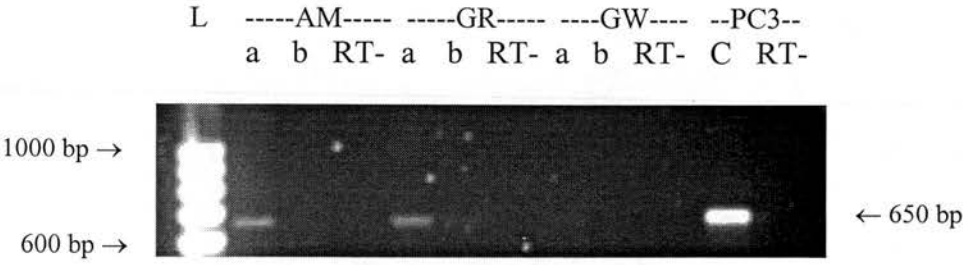
## 4.1 Oestrogen Receptor mRNA Expression in Prostatic Cells in Primary Culture

A number of previous studies have reported the expression of both oestrogen receptor subtypes (ER $\alpha$  and ER $\beta$ ) in the human prostate; however, results from different groups do not entirely agree with one another (Ehara et al. 1995, Bonkhoff et al. 1999, Royuela et al. 2001, Pasquali et al. 2001a). In this study, to determine if prostatic cells in primary culture have the potential to respond to oestrogen treatment, total RNA was prepared from epithelial cells and fibroblasts derived from 3 BPH patients and analysed for ER $\alpha$  and ER $\beta$  mRNA expression using RT-PCR. Also, to determine whether ER mRNA expression is affected by the process of cell passaging, serial dilutions of fibroblast (passage numbers 1-5) RT reactions were used in the PCR reactions. Although the PCR used was not quantitative, the results obtained from serial dilutions of RT reactions give an indication as to the relative levels of mRNA expression among fibroblasts at different passage numbers. All RT reactions were carried out under identical conditions at the same time. Epithelial cells of passage number 1 only were used because they did not grow well after the second passage.

### 4.1.1 ER $\alpha$ mRNA expression in prostatic cells in primary culture

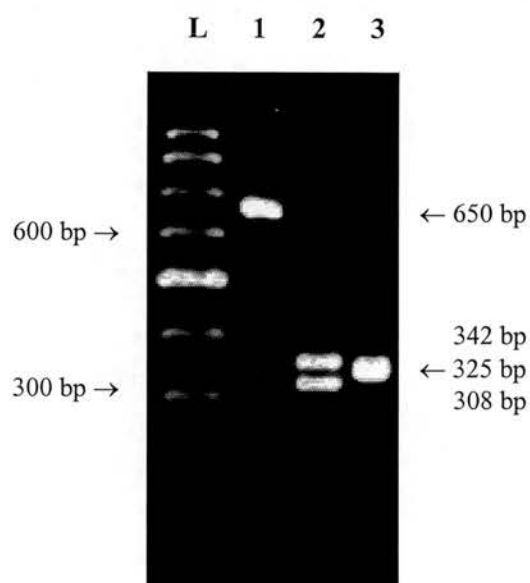
#### 4.1.1a Epithelial cells

Of the three samples of epithelial cell RNA tested, two (patients AM and GR) expressed ER $\alpha$  mRNA (fig. 4.1 & table 4.1). However, no RT-PCR product was detectable when 10-fold diluted RT reactions were used, suggesting relatively low levels of ER $\alpha$  mRNA expression in these cells. Identity of the 650-bp PCR product was confirmed with restriction enzymes *Pvu*II and *Pst*I (fig. 4.2).



**Fig. 4.1 ER $\alpha$  mRNA is expressed in epithelial cells cultured from BPH tissues.**

Total RNA was prepared from epithelial cells (passage number 1) of 3 different BPH patients (AM, GR, GW) and from PC3 cells. The same amount of RNA from each sample underwent RT at the same time under identical conditions. PCR reactions (40 cycles) were carried out on (a) undiluted, (b) 10-fold diluted or 100-fold diluted (not shown) RT reactions. RT-, negative control in which reverse transcriptase was omitted; C, positive control using RNA isolated from PC3 cells. L, 100-bp DNA ladder.



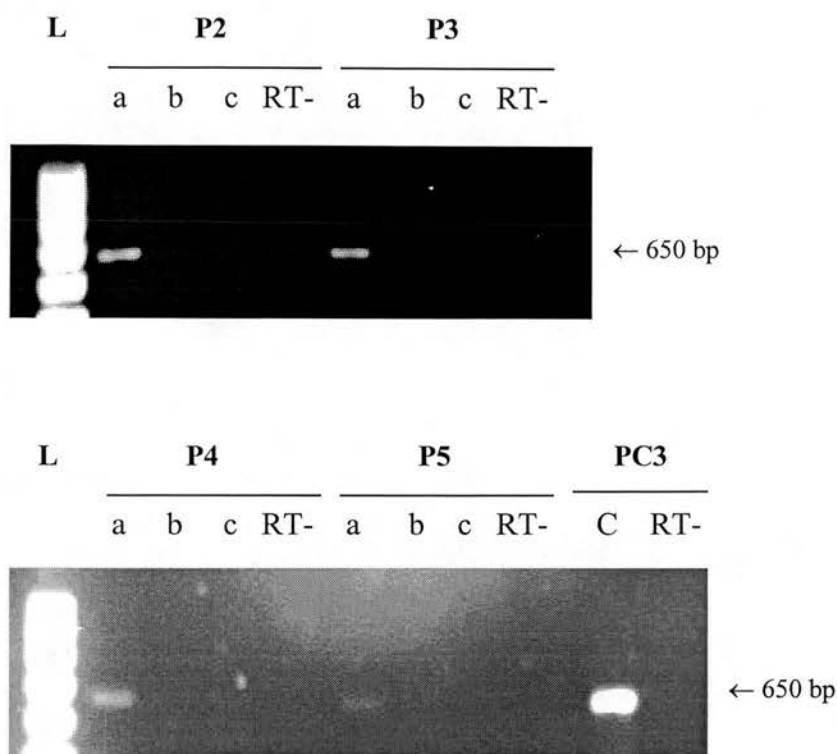
**Fig. 4.2 Restriction enzyme digestion of ER $\alpha$  PCR product.** The 650-bp ER $\alpha$  PCR product was digested with two restriction enzymes to confirm its identity. Lane 1, undigested PCR product; lane 2, *PvuII* produced two fragments of 308 and 342 bp; lane 3, *PstI* produced two fragments of the same size, 325 bp. L, 100-bp DNA ladder.

#### 4.1.1b Fibroblasts

Fibroblasts (passage numbers 1 to 5) derived from all 3 BPH patients expressed ER $\alpha$  mRNA (fig. 4.3 and table 4.1). Again, PCR product was only detectable using undiluted RT reactions, suggesting that the levels of mRNA expression in these cells were also relatively low.

		<b>AM</b>			<b>GR</b>			<b>GW</b>		
		cDNA dilution			cDNA dilution			cDNA dilution		
Cell type	Passage number	1:1	1:10	1:100	1:1	1:10	1:100	1:1	1:10	1:100
<b>Ep</b>	P1	+	-	-	+	-	-	-	-	-
<b>Fb</b>	P1	+	-	-	nt	nt	nt	+	-	-
	P2	+	-	-	+	-	-	+	-	-
	P3	+	-	-	+	-	-	+	-	-
	P4	+	-	-	+	-	-	+	-	-
	P5	+	-	-	+	-	-	+	-	-

Table 4.1 ER $\alpha$  mRNA expression in epithelial cells and fibroblasts in primary culture. Total RNA was prepared from epithelial cells (Ep, passage number 1) and fibroblasts (Fb, passage number up to 5) derived from three BPH tissue samples (designated AM, GR and GW). PCR reactions (40 cycles) were carried out using RT reactions diluted up to 100-fold. +, present; -, absent; nt, not tested. PCR was repeated once on the undiluted epithelial cell RT reaction (patient GW) and again yielded no PCR product.



**Fig. 4.3 ER $\alpha$  mRNA is expressed in fibroblasts cultured from BPH tissues.** Total RNA was prepared from fibroblasts (passage numbers 2 to 5) of BPH patient GR and from PC3 cells. The same amount of RNA from each sample underwent RT at the same time under identical conditions. PCR reactions (40 cycles) were carried out on (a) undiluted, (b) 10-fold diluted, and (c) 100-fold diluted RT reactions. L, 100-bp DNA ladder; P2-P5, fibroblasts of passage numbers 2 to 5; RT-, negative control in which reverse transcriptase was omitted; C, positive control using RNA isolated from PC3 cells. RT-PCR reactions carried out on total RNA prepared from two other patients' fibroblasts generated similar results (table 4.1).

## 4.1.2 ER $\beta$ mRNA expression in prostatic cells in primary culture

### 4.1.2a Epithelial cells

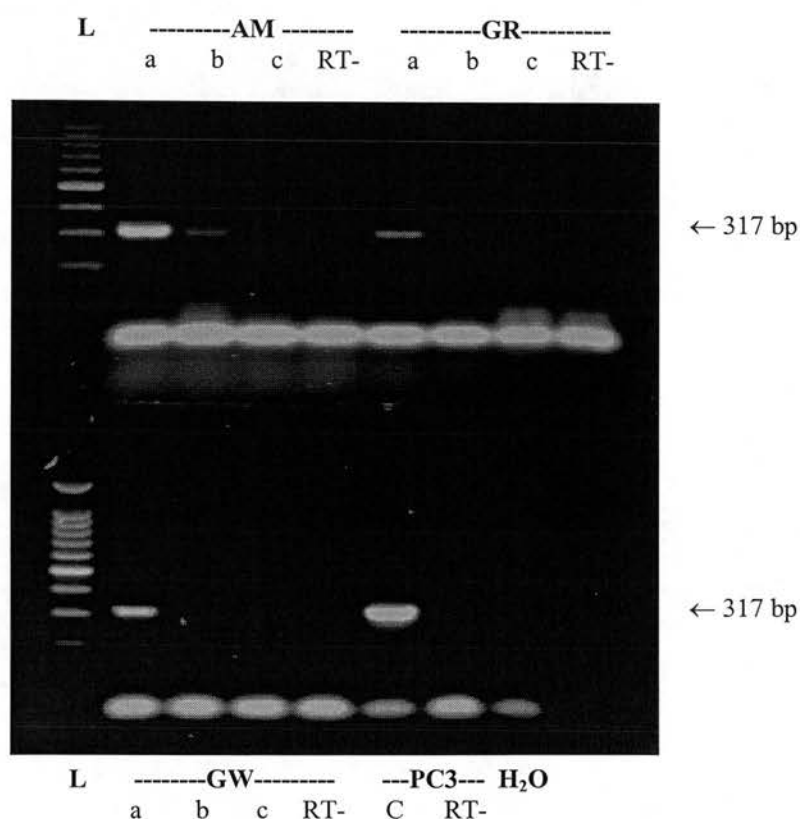
All 3 samples of epithelial cells expressed ER $\beta$  mRNA (fig. 4.4 & table 4.2). PCR product was detectable with all 3 undiluted RT reactions and only 1 of the 3 samples of 10-fold diluted RT reactions. Identity of the 317-bp PCR product was confirmed with restriction enzymes *DraI* and *HinfI* (fig. 4.5).

### 4.1.2b Fibroblasts

Similarly, all 3 samples of fibroblasts (passage numbers 1 to 5) expressed ER $\beta$  mRNA (fig. 4.6 & table 4.2). PCR product was detectable with all of the undiluted RT reactions, some of the 10-fold diluted RT reactions and none of the 100-fold diluted RT reactions. With increasing passage numbers from 1 to 5, there is no clear pattern of up- or down-regulation of ER $\beta$  mRNA expression in fibroblasts.

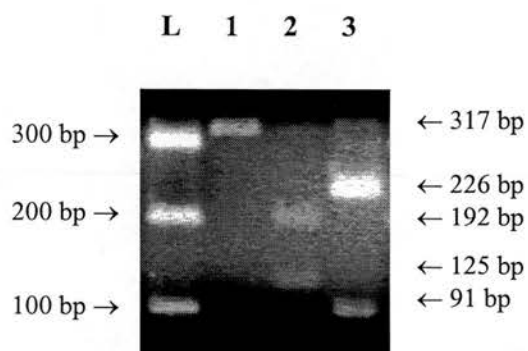
		AM			GR			GW		
		cDNA dilution			cDNA dilution			cDNA dilution		
Cell type	Passage number	1:1	1:10	1:100	1:1	1:10	1:100	1:1	1:10	1:100
<b>Ep</b>	P1	+	+	-	+	-	-	+	-	-
<b>Fb</b>	P1	+	+	-	nt	nt	nt	+	+	-
	P2	+	+	-	+	-	-	+	+	-
	P3	+	+	-	+	+	-	+	+	-
	P4	+	+	-	+	+	-	+	+	-
	P5	+	-	-	+	+	-	+	+	-

Table 4.2 ER $\beta$  mRNA is expressed in epithelial cells and fibroblasts in primary culture. Total RNA was prepared from epithelial cells (Ep, passage number 1) and fibroblasts (Fb, passage number up to 5) derived from three BPH tissue samples (designated AM, GR and GW). PCR reactions (40 cycles) were carried out using RT reactions diluted up to 100-fold. +, present; -, absent; nt, not tested.



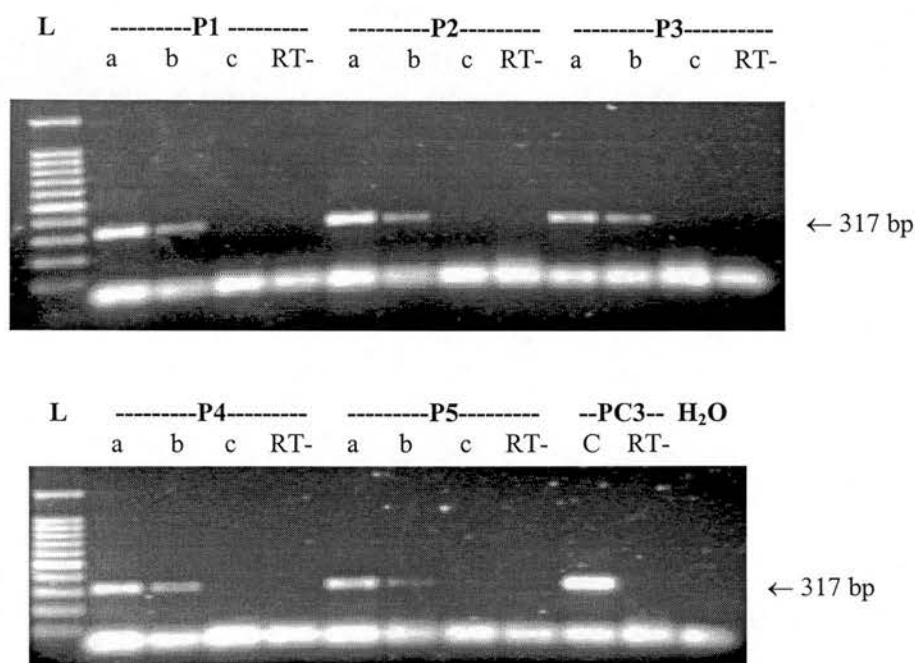
**Fig. 4.4 ER $\beta$  mRNA is expressed in epithelial cells cultured from BPH tissues.**

Total RNA was prepared from epithelial cells (passage number 1) of 3 different BPH patients (AM, GR, GW) and from PC3 cells. The same amount of RNA from each sample underwent RT at the same time under identical conditions. PCR reactions (40 cycles) were carried out on (a) undiluted, (b) 10-fold diluted or (c) 100-fold diluted RT reactions. RT-, negative control in which reverse transcriptase was omitted; C, positive control using RNA isolated from PC3 cells. L, 100-bp DNA ladder; H<sub>2</sub>O, negative PCR control with water in place of RT reaction.



**Fig. 4.5 Restriction enzyme digestion of ER $\beta$  PCR product.** The 317-bp ER $\beta$  PCR product was digested with two restriction enzymes to confirm its identity. Lane 1, undigested PCR product; lane 2, *DraI* produced two fragments of 125 and 192 bp; lane 3, *HinfI* produced two fragments of 91 and 226 bp. L, 100-bp DNA ladder.





**Fig. 4.6 ER $\beta$  mRNA is expressed in fibroblasts cultured from BPH tissues.** Total RNA was prepared from fibroblasts (passage numbers 1 to 5) of BPH patient GW and from PC3 cells. The same amount of RNA from each sample underwent RT at the same time under identical conditions. PCR reactions (40 cycles) were carried out on (a) undiluted, (b) 10-fold diluted, and (c) 100-fold diluted RT reactions. L, 100-bp DNA ladder; P1-P5, fibroblasts of passage numbers 1 to 5; RT-, negative control in which reverse transcriptase was omitted; C, positive control using RNA isolated from PC3 cells. H<sub>2</sub>O, negative PCR control with water in place of RT reaction.

#### 4.1.3 Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA confirms the integrity of RNA samples

The results shown in tables 4.1 and 4.2 reveal that most of the PCR products became undetectable when RT reactions were diluted by 10- or 100-fold. To confirm that the variations in the dilution factors at which PCR products became undetectable were not due to degradation of RNA or cDNA, the RT reactions were subjected to PCR using primers for the housekeeping gene GAPDH (data summarised in table 4.3). The GAPDH PCR product was clearly detectable in all dilutions up to  $10^4$ -fold, verifying that the lack of ER $\alpha$  or ER $\beta$  RT-PCR product in the corresponding dilutions was not due to RNA/cDNA degradation. Moreover, there is no correlation among results in tables 4.1, 4.2 and 4.3, suggesting that variations in ER PCR product reported are not due to variations in the amount of RNA used.

		AM			GR			GW		
		cDNA dilution			cDNA dilution			cDNA dilution		
Cell type	Passage number	1:10 <sup>4</sup>	1:10 <sup>5</sup>	1:10 <sup>6</sup>	1:10 <sup>4</sup>	1:10 <sup>5</sup>	1:10 <sup>6</sup>	1:10 <sup>4</sup>	1:10 <sup>5</sup>	1:10 <sup>6</sup>
<b>Ep</b>	P1	+	+	-	+	+	-	+	-	-
<b>Fb</b>	P1	+	+	-	nt	nt	nt	+	+	-
	P2	+	-	-	+	+	-	+	+	-
	P3	+	+	-	+	+	-	+	-	-
	P4	+	+	-	+	+	-	+	-	-
	P5	+	+	-	+	+	-	+	+	-

Table 4.3 GAPDH mRNA expression in epithelial cells and fibroblasts prepared from three BPH tissues. Total RNA was prepared from epithelial cells (Ep, passage number 1) and fibroblasts (Fb, passage number up to 5) derived from three BPH tissue samples (designated AM, GR and GW). PCR reactions (40 cycles) were carried out using RT reactions diluted up to  $10^6$ -fold. +, present; -, absent; nt, not tested.

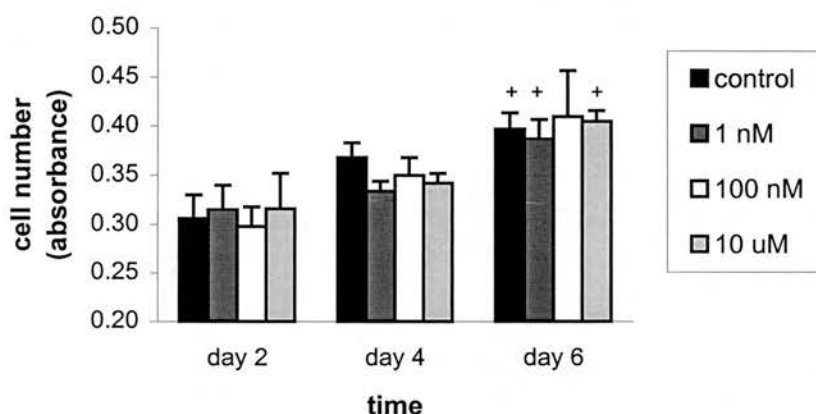
## 4.2 Growth Response of Prostatic Cells in Primary Culture to Oestrogens

The results described above show that both epithelial cells and fibroblasts in primary culture express mRNA of both ER subtypes, which are essential for oestrogen action. Both cell types are therefore potential oestrogen target cells. BPH results from excessive cell proliferation, diminished cell death or a combination of both. To ascertain whether oestrogens play a role in BPH pathogenesis, the next part of the present study aimed to examine the effects of oestrogens on prostatic cell proliferation using prostatic cells in primary culture and the MTS assay described in section 2.2.4.

Recently, Weihua et al. (2001) suggested a role for 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ Adiol) in prostate oestrogenic response in the mouse. Although 3 $\beta$ Adiol has low binding affinities for both ER subtypes, its concentrations in the human prostate are high compared with those of E<sub>1</sub> and E<sub>2</sub> (table 1.7, section 1.7.3). Also, the potent androgen DHT can be converted to 3 $\beta$ Adiol by 3 $\beta$ -hydroxysteroid dehydrogenase within the prostate (Krieg et al. 1995). Thus, 3 $\beta$ Adiol may have a potential role in the androgen/oestrogen balance within the prostate gland. To this end, effects of this putative oestrogen on human prostatic cell proliferation were also investigated and compared with those of oestradiol.

### 4.2.1 Epithelial cells

Figure 4.7 shows that E<sub>2</sub> treatment (1 nM - 10  $\mu$ M) had no effect on the growth of epithelial cells (passage number 1) compared with control cells with up to 6 d incubation. Similarly, 3 $\beta$ Adiol treatment (1 nM - 10  $\mu$ M) did not affect epithelial cell proliferation (data not shown).



**Fig 4.7 Oestradiol had no effect on epithelial cell growth.** 3000 cells/well in serum-free EGM were seeded into 96-well plates 2 d before the addition of E<sub>2</sub> (1 nM – 10  $\mu$ M, 6 wells per treatment group). 6 control wells received 1% ethanol (v/v) to control for the ethanol used as a vehicle for E<sub>2</sub>. Medium was changed daily. MTS assay was performed after 2, 4 or 6 d of E<sub>2</sub> treatment. Values are expressed as mean  $\pm$  sem. There is no statistical significance between the various treatments groups on the same day. <sup>+</sup>P<0.05 compared with the same treatment/control group on day 2. Results are representative of 3 separate experiments using epithelial cells derived from 4 different BPH patients.

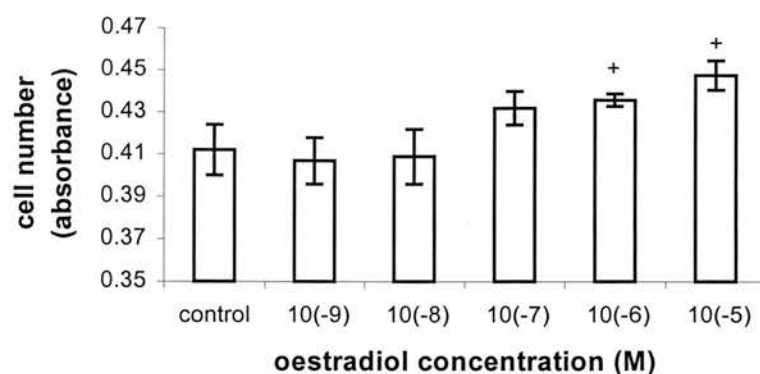
#### 4.2.2 Fibroblasts

In contrast to the absence of effect on epithelial cell proliferation, E<sub>2</sub> (1  $\mu$ M and 10  $\mu$ M) stimulated the proliferation of fibroblasts grown in phenol red-free RPMI supplemented with 10% DCC-FCS (fig. 4.8a). Similarly, at high concentrations (1  $\mu$ M and 10  $\mu$ M), 3 $\beta$ Adiol also stimulated the growth of fibroblasts grown in medium supplemented with 10% DCC-FCS (fig. 4.8b).

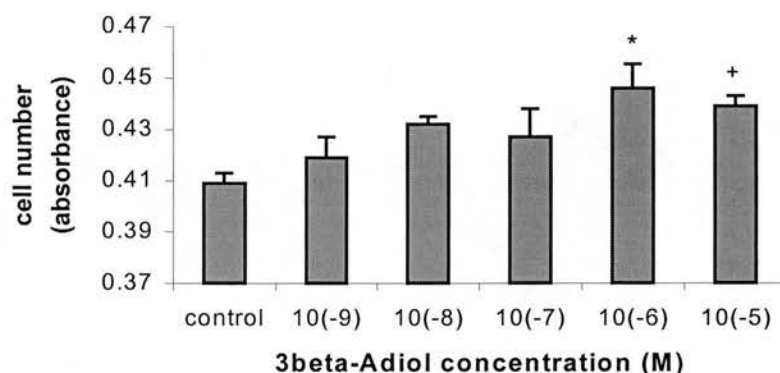
The micromolar concentrations of E<sub>2</sub> and 3 $\beta$ Adiol required to stimulate fibroblast proliferation are much higher than their physiological concentrations (<10 nM; table 1.7, p.36). One possible explanation is that growth factors present in DCC-stripped serum are masking the stimulatory effects of both oestrogenic compounds. Accordingly, further experiments were carried out using medium supplemented with reduced amount of DCC-FCS.

A preliminary experiment was set up to determine the lowest concentration of DCC-FCS suitable for fibroblast proliferation experiments (fig. 4.9). Fibroblasts were seeded into 96-well plates in triplicate and supplemented with various concentrations of DCC-FCS (0-10%, v/v). After 48 or 96 h incubation in medium containing 0% or 1% DCC-FCS, microscopic examination of fibroblasts revealed rounded cells, rather than the normal spindle-shaped fibroblasts (data not shown). With 2% DCC-FCS concentration and above, fibroblasts not only were spindle-shaped but also demonstrated proliferation between 48 h and 96 h (fig. 4.9). With increasing concentrations of DCC-FCS from 1% to 10%, the difference in cell numbers between 48 h and 96 h after initial plating also increased. To minimise the background stimulation of fibroblast proliferation by DCC-FCS and yet sustain continuous growth of fibroblasts in primary culture, 2% DCC-FCS supplement was used in subsequent oestrogenic response experiments.

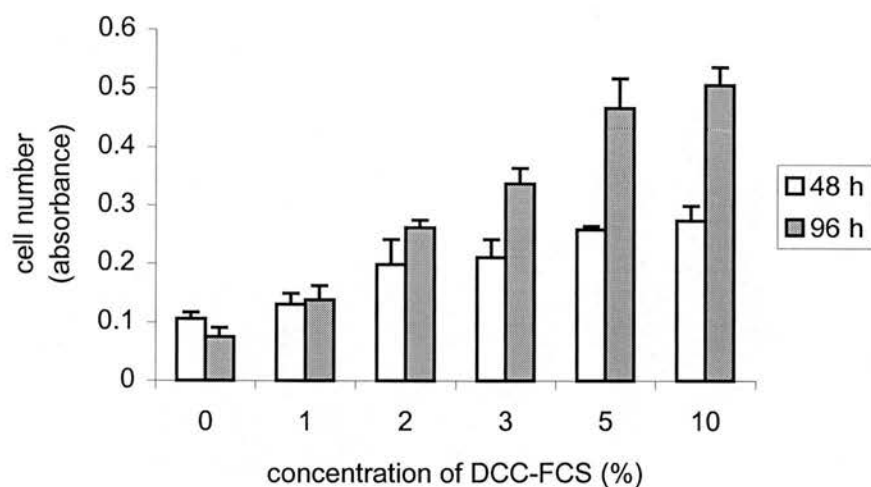
(a) E<sub>2</sub> treatment



(b) 3 $\beta$ Adiol treatment



**Fig. 4.8 Effects of oestradiol and 3 $\beta$ Adiol on fibroblast growth in medium supplemented with 10% DCC-FCS.** 1800 cells/well in phenol red-free RPMI supplemented with 10% DCC-FCS were seeded into 96-well plates 2 d before the addition of  $10^{-9}$  –  $10^{-5}$  M of (a) E<sub>2</sub> or (b) 3 $\beta$ Adiol (6 wells per treatment group). 6 wells received 1% ethanol (v/v) to control for the ethanol used as a vehicle for E<sub>2</sub> or 3 $\beta$ Adiol. Medium was changed daily. MTS assay was performed 4 d later. Values are expressed as mean  $\pm$  sem. <sup>+</sup>P<0.05, \*P<0.01 compared with controls. Results are representative of 3 experiments using fibroblasts derived from 3 BPH patients.



**Fig 4.9 Effects of the concentration of DCC-FCS on fibroblast proliferation.** 1800 fibroblasts/well in phenol red-free RPMI supplemented with 0-10% DCC-FCS (v/v) were seeded into 96-well plates in triplicate. Medium was changed daily. MTS assay was performed 48 h or 96 h after initial plating of cells. Results are expressed as mean of triplicates  $\pm$  s.d.

Figure 4.10a shows that E<sub>2</sub> at concentrations between 10 nM and 1 µM stimulated the proliferation of fibroblasts grown in medium containing 2% DCC-FCS, with maximal effect at 100 nM. This is in contrast to the high E<sub>2</sub> concentrations required to stimulate fibroblasts grown in medium supplemented with 10% DCC-FCS (fig. 4.8a). Similarly, 3βAdiol (100 nM - 10 µM) also stimulated the proliferation of fibroblasts grown in medium containing 2% DCC-FCS with maximal effect at 100 nM (fig. 4.10b).

In summary, the above results show that both E<sub>2</sub> and 3βAdiol stimulated fibroblast proliferation. Whereas micromolar concentrations of both compounds were required to stimulate proliferation of fibroblasts grown in medium supplemented with 10% DCC-FCS, lower (nanomolar) concentrations increased fibroblast proliferation in medium containing 2% DCC-FCS only.

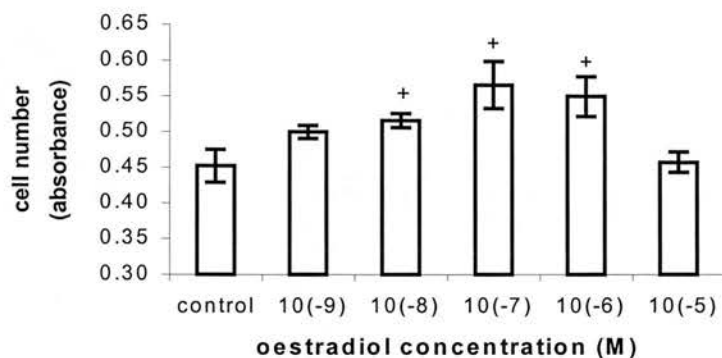
#### **4.3 Stimulatory Effects of E<sub>2</sub> and 3βAdiol on Prostatic Fibroblasts in Primary Culture are Antagonised by the Anti-oestrogen ICI 182780**

To determine if the stimulatory effects of both E<sub>2</sub> and 3βAdiol on fibroblast growth were oestrogen receptor-mediated, the anti-oestrogen ICI 182780 was added to fibroblasts grown in medium supplemented with 2% DCC-FCS. ICI 182780 (Tochris, Bristol, UK), a pure anti-oestrogen, competitively inhibits E<sub>2</sub> binding to oestrogen receptor; it does not exhibit agonist or partial agonist actions *in vivo* or *in vitro* (reviewed in MacGregor & Jordan 1998). ICI 182780 (Faslodex<sup>®</sup>) is currently being tested in clinical trials for the treatment of breast cancer.

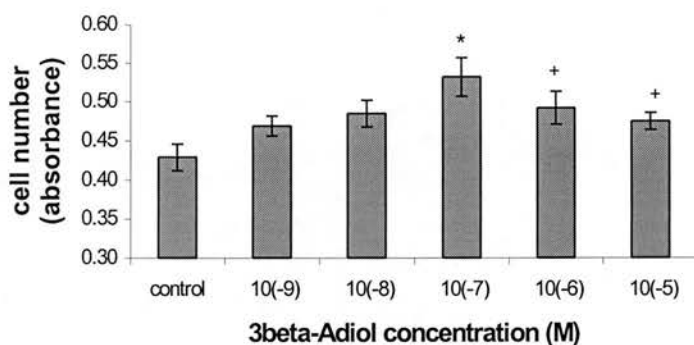
Preliminary experiments were carried out on fibroblasts growing in phenol red-free RPMI medium supplemented with 2% DCC-FCS and various concentrations of ICI 182780 but no oestrogens. Treatment with up to 100 nM ICI 182780 had no effect on fibroblast proliferation. However, 1 µM ICI 182780 inhibited fibroblast proliferation by approximately 15% after 2 d incubation (fig. 4.11).



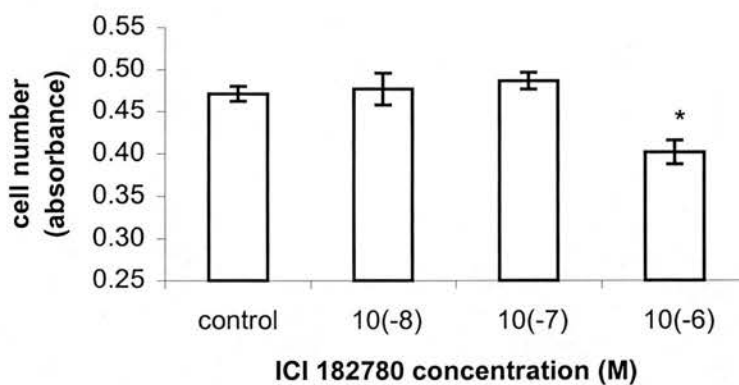
(a) E<sub>2</sub> treatment



(b) 3 $\beta$ Adiol treatment



**Fig. 4.10 Effects of oestradiol and 3 $\beta$ Adiol on fibroblast growth in medium supplemented with 2% DCC-FCS.** 1800 fibroblasts/well in phenol red-free RPMI supplemented with 2% DCC-FCS were seeded into 96-well plates 2 d before the addition of  $10^{-9}$  –  $10^{-5}$  M of (a) E<sub>2</sub> or (b) 3 $\beta$ Adiol (6 wells per treatment group). 6 control wells received 1% ethanol (v/v) to control for the ethanol used as a vehicle for E<sub>2</sub> or 3 $\beta$ Adiol. Medium was changed daily. MTS assay was performed 4 d after initial plating of cells. Values are expressed as mean  $\pm$  sem. <sup>+</sup>P<0.05, <sup>\*</sup>P<0.01 compared with controls. Results are representative of 3 experiments using fibroblasts derived from 3 BPH patients.



**Fig 4.11 The anti-oestrogen ICI 182780 inhibits the proliferation of prostatic fibroblasts.** 1800 fibroblasts/well in phenol red-free RPMI supplemented with 2% DCC-FCS were seeded into 96-well plates 2 d before the addition of  $10^{-8}$  –  $10^{-6}$  M ICI 182780 (6 wells per treatment group). 6 control wells received 1% ethanol (v/v) to control for the ethanol used as a vehicle for ICI 182780. Medium was changed daily. MTS assay was performed 4 d after initial plating of cells. Values are expressed as mean  $\pm$  sem. \* $P < 0.01$  compared with controls. Results are representative of 2 experiments using fibroblasts derived from 2 BPH patients.

In order to establish whether the fibroblast proliferation stimulated by E<sub>2</sub> and 3βAdiol was ER-mediated, and to rule out any effect on fibroblast growth by the anti-oestrogen on its own, ICI 182780 concentrations less than 100 nM were used in subsequent experiments involving both oestrogens and ICI 182780.

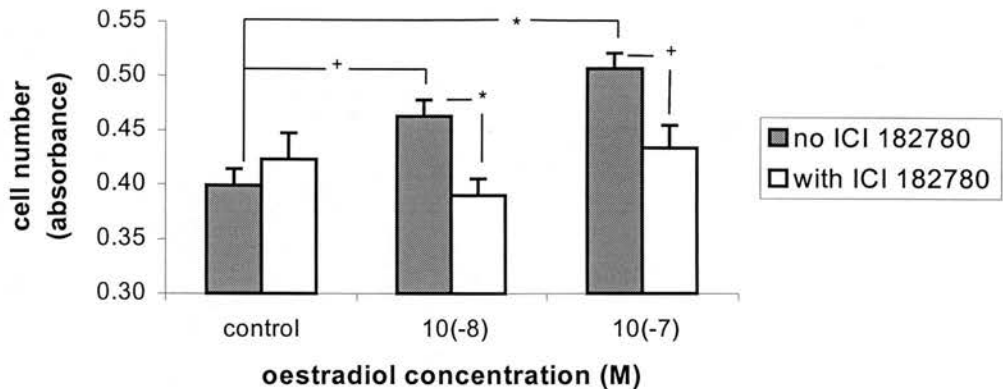
Figure 4.12 clearly shows that 50 nM ICI 182780 had no effect on fibroblast growth in the absence of E<sub>2</sub> or 3βAdiol. In fibroblasts without any anti-oestrogen treatment, E<sub>2</sub> (10 & 100 nM) and 3βAdiol (100 nM) increased cell proliferation; these stimulatory effects of both E<sub>2</sub> and 3βAdiol were antagonised by 50 nM ICI 182780, confirming that such effects were ER-mediated.

#### **4.4 Oestrogen Catabolism in Prostatic Cells in Primary Culture**

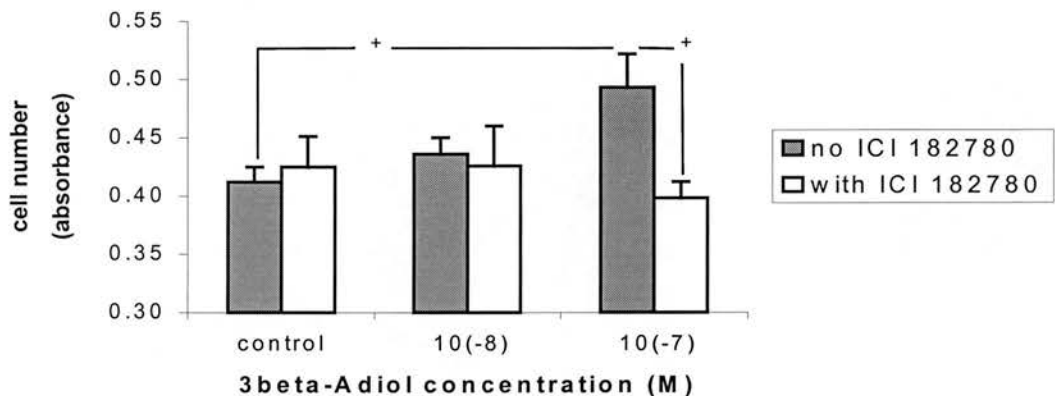
The above data demonstrated that cell growth of prostatic fibroblasts, but not epithelial cells, was stimulated by E<sub>2</sub> and 3βAdiol. At the beginning of this chapter, it was hypothesised that prostatic cells are not only oestrogen target cells but also capable of modulating the effects of oestrogens by active oestrogen metabolism. In Chapter 3, it was found that fibroblasts in primary culture expressed aromatase mRNA and enzyme activity; fibroblasts are thus capable of local biosynthesis of oestrogens from androgen precursors (oestrogen anabolism). More experiments were carried out to investigate if prostatic cells in primary culture can further metabolise oestrogens (oestrogen catabolism) and regulate the local concentrations and effects of oestrogens in the prostate.

No previous study has reported the conversion of oestrogens to their metabolites in prostatic cells. To establish if these cells can metabolise the natural oestrogens E<sub>2</sub> and E<sub>1</sub>, preliminary time-course experiments were carried out by incubation of epithelial cells and fibroblasts in primary culture with 10 nM radiolabelled E<sub>2</sub> or E<sub>1</sub> (fig. 4.13).

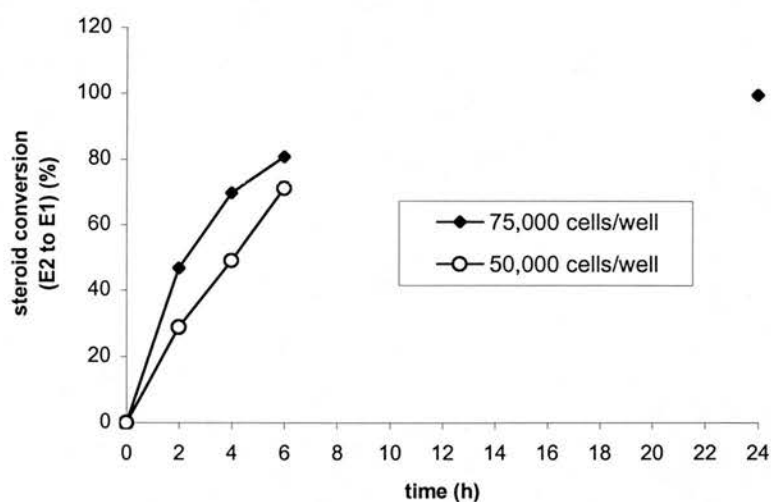
(a) E<sub>2</sub> and ICI 182780



(b) 3 $\beta$ Adiol and ICI 182780



**Fig. 4.12 Stimulatory effects of oestradiol and 3 $\beta$ Adiol on fibroblast growth are antagonised by the anti-oestrogen ICI 182780.** 1800 fibroblasts/well in phenol red-free RPMI supplemented with 2% DCC-FCS were seeded into 96-well plates 2 d before the addition of 10<sup>-8</sup> or 10<sup>-7</sup> M (a) E<sub>2</sub> or (b) 3 $\beta$ Adiol (6 wells per treatment group) with or without 50 nM ICI 182780. 6 wells received 1% ethanol (v/v) to control for the ethanol used as a vehicle for E<sub>2</sub>, 3 $\beta$ Adiol and ICI 182780. Medium was changed daily. MTS assay was performed 4 d after initial plating of cells. Values are expressed as mean  $\pm$  sem. <sup>+</sup>P<0.05, \*P<0.01 compared with controls. Results are representative of 3 experiments using fibroblasts derived from 3 BPH patients.



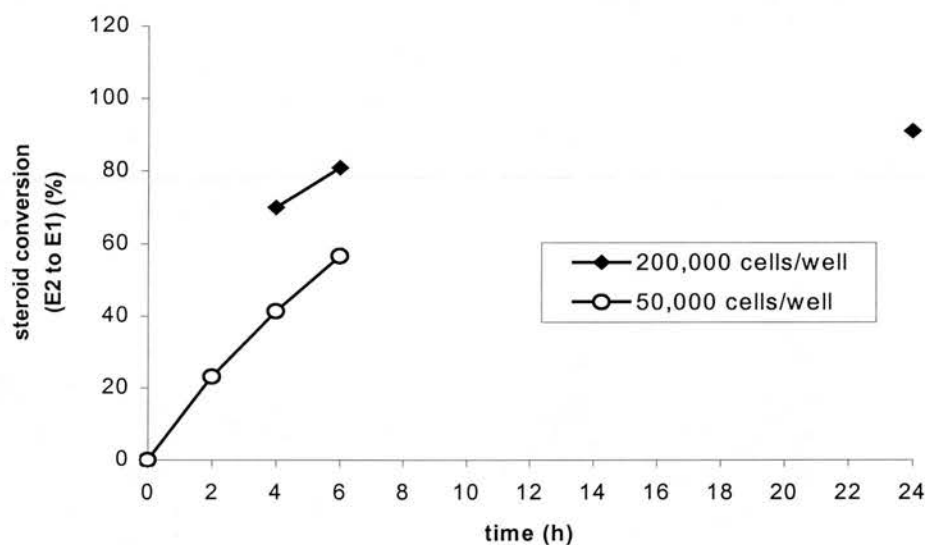
**Fig 4.13 Time course of oestradiol ( $E_2$ ) oxidation to oestrone ( $E_1$ ) by prostatic epithelial cells in primary culture.** Either 50,000 or 75,000 epithelial cells/well in serum-free EGM were plated in duplicate in 6-well plates. 48 h later, the cells were incubated with 2 ml of the same medium containing 10 nM of radiolabelled  $E_2$  for up to 24 h. Steroid metabolites were extracted from the medium and analysed by HPLC as described in sections 2.6.2 & 2.7.2. Results are expressed as mean of duplicates.

Figure 4.13 shows that with epithelial cells in primary culture, initial plating density of  $7.5 \times 10^4$  cells/well resulted in rapid conversion of approximately 50% of  $E_2$  to  $E_1$  within 2 h of incubation. At a reduced plating density of  $5 \times 10^4$  epithelial cells/well,  $E_2$  metabolism to  $E_1$  was more or less proportional with time up to 6 h. No other steroid metabolites were detectable by HPLC within 24 h of incubation. In similar experiments using  $E_1$  as substrate, no  $E_2$  or other metabolite was formed within 24 h of incubation (data not shown).

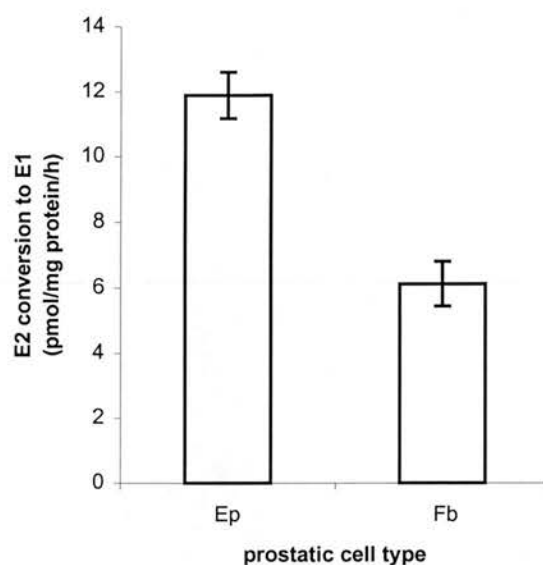
Similar to epithelial cells, fibroblasts at a plating density of  $20 \times 10^4$  cells/well rapidly catabolised  $E_2$  to  $E_1$ . At a reduced plating density of  $5 \times 10^4$  cells/well, fibroblast metabolism of  $E_2$  to  $E_1$  was essentially proportional with time in up to 6 h (fig. 4.14). Again, no other steroid metabolites were detectable by HPLC within 24 h of incubation. In similar experiments using  $E_1$  as substrate, no  $E_2$  or other metabolite was formed within 24 h of incubation (data not shown).

In summary, both cell types converted the most potent natural oestrogen,  $E_2$ , to the weaker  $E_1$  (oxidative  $17\beta$ HSD activity) but not vice versa.  $E_1$  was not further metabolised by either epithelial cells or fibroblasts using the current culture conditions. After ethyl acetate extraction of steroids from the culture medium, scintillation counting of radioactivity remaining in the aqueous phase revealed no difference among cells incubated for 0, 2, 4, 6 and 24 h, indicating that neither cell type conjugated  $E_2$  or  $E_1$  to water-soluble metabolites.

In subsequent experiments to compare oxidative  $17\beta$ HSD activities (oestrogen catabolism) in epithelial cells and fibroblasts in primary culture, cells were plated at a density of  $5 \times 10^4$  cells/well 48 h before incubation with radiolabelled  $E_2$  for 4 h. Conversion rate of  $E_2$  to  $E_1$  in fibroblasts ( $6.12 \pm 0.68$  pmol/mg protein/h) was approximately 50% less than that in epithelial cells ( $11.89 \pm 0.72$  pmol/mg protein/h) (fig. 4.15).



**Fig 4.14 Time course of oestradiol ( $E_2$ ) oxidation to oestrone ( $E_1$ ) by prostatic fibroblasts in primary culture.** Either 50,000 or 200,000 fibroblasts/well in phenol red-free RPMI containing 10% DCC-FCS were plated in duplicate in 6-well plates. 48 h later, the cells were incubated with 2 ml of the same medium containing 10 nM of radiolabelled  $E_2$  for up to 24 h. Steroid metabolites were extracted from the medium and analysed by HPLC as described in sections 2.6.2 & 2.7.2. Results are expressed as mean of duplicates.



**Fig 4.15 Comparison of oestradiol catabolism by  $17\beta$ HSD in epithelial cells and fibroblasts.** 50,000 cells/well were plated in triplicate in 6-well plates. 48 h later, the cells were incubated with 10 nM radiolabelled  $E_2$  in 2 ml of either serum-free EGM for epithelial cells (Ep) or phenol red-free RPMI supplemented with 10% DCC-FCS for fibroblasts (Fb). Medium was collected after 4 h incubation with  $E_2$ ; steroid metabolites were extracted from medium and analysed by HPLC. Cells were then lysed and protein concentrations determined as described in sections 2.6.2 & 2.3.4 respectively. Results are expressed as mean  $\pm$  s.e.m. (n=9) from 3 separate experiments using epithelial cells (passage number 1) and fibroblasts (passage number 2 or 3) from 3 BPH patients.



## 4.5 Discussion

At the beginning of this chapter, it was hypothesised that prostatic cells not only are oestrogen target cells but can also modulate the effects of oestrogens. A number of oestrogen response parameters, including mRNA encoding both oestrogen receptor subtypes and enzymes responsible for biosynthesis (aromatase) and catabolism (17 $\beta$ HSD) of oestrogens, were shown to be present in prostatic cells in primary culture. Moreover, E<sub>2</sub> and 3 $\beta$ Adiol stimulated the proliferation of prostatic fibroblasts but not epithelial cells; these effects on fibroblasts in primary culture were antagonised by the anti-oestrogen ICI 182780.

Although expression and localisation of both ER $\alpha$  and ER $\beta$  in the human prostate is still unresolved, previous *in situ* mRNA hybridisation and immunohistochemical studies have localised ER $\alpha$  expression mainly, but not exclusively, to the stroma, whereas ER $\beta$  was expressed predominantly in the human prostatic epithelium (section 1.6.4). In the present study, ER $\alpha$  mRNA was present in 2 out of 3 samples of BPH-derived epithelial cells in primary culture, whereas all 3 samples contained ER $\beta$  mRNA. Using RT-PCR, Lau et al. (2000) detected ER $\beta$  mRNA expression in all 5 primary cultures of normal prostate epithelial cells grown from patients undergoing radical cystectomy because of bladder cancer; however, none of the 5 samples contained ER $\alpha$  mRNA. In contrast, another study reported mRNA encoding both ER subtypes in all of 6 primary cultures of normal prostate epithelial cells from ultrasound-guided peripheral zone prostate biopsies (Pasquali et al. 2001b). Taken together, it appears that ER $\beta$  mRNA is consistently expressed in epithelial cells in primary culture, while the expression of ER $\alpha$  mRNA is more variable (summarised in table 4.4).

All 3 prostatic fibroblast samples tested in this study contained both ER $\alpha$  and ER $\beta$  mRNA. Only one previous study has looked at ER mRNA expression in fibroblasts in primary culture; all 6 fibroblast samples collected from patients

undergoing radical cystectomy because of bladder cancer expressed ER $\alpha$  but not ER $\beta$  mRNA (Pasquali et al. 2001b). To summarise, all studies to date on both prostatic tissues and primary cultures found ER $\alpha$  expression in stromal cells whereas ER $\beta$  expression in these cells is still unresolved.

	Lau et al. (2000)	Pasquali et al. (2001b)		present study	
	Ep	Ep	Fb	Ep	Fb
ER $\alpha$	-	+	+	$\pm$	+
ER $\beta$	+	+	-	+	+

Table 4.4 Expression of ER mRNA in prostatic cells in primary culture as detected by RT-PCR. Ep, epithelial cells; Fb, fibroblasts. +, present; -, absent;  $\pm$ , present in 2 of 3 samples.

Although all 3 samples of epithelial cells examined in this study expressed mRNA encoding either or both oestrogen receptor subtypes, they did not display any growth response to E<sub>2</sub> or 3 $\beta$ Adiol treatment up to 10  $\mu$ M. This observation is analogous to a previous study in which epithelial cells in primary culture expressed androgen receptor mRNA detectable by RT-PCR but did not show any growth response to DHT stimulation (Grant et al. 1996). It is possible that the level of ER gene expression in epithelial cells in the present study was insufficient to provide the cells with enough receptor protein to effect any growth response to oestrogen stimulation. An alternative explanation is that E<sub>2</sub> and 3 $\beta$ Adiol had no effect on epithelial cell proliferation but affect other oestrogen responsive gene expression such as progesterone receptor. A third possibility is that the growth-stimulating substances present in the epithelial growth medium, including insulin and epidermal growth factor, were masking the effects of oestrogens on these cells.

In contrast, proliferation of prostatic fibroblasts in primary culture was stimulated by both E<sub>2</sub> and 3 $\beta$ Adiol. Two previous studies have reported the effects of E<sub>2</sub> on BPH-derived fibroblasts in culture. In one study, E<sub>2</sub> (10 pM – 100 nM) failed to stimulate proliferation of prostatic fibroblasts grown in medium supplemented with 0% or 1% FCS (Levine et al. 1992). In another study, E<sub>2</sub> increased fibroblast proliferation grown in medium containing 0.5% FCS in a dose-dependent manner with maximal stimulation at 1 nM; this stimulatory effect was antagonised by the anti-oestrogen tamoxifen (Collins et al. 1994). In comparison, in an animal model of prostate adenocarcinoma in which rats were castrated and supplemented with testosterone, additional E<sub>2</sub> treatment increased the volume of the stromal compartment but decreased that of the epithelial compartment (Daehlin et al. 1987). Taken together, results presented in this thesis and from previous *in vitro* and *in vivo* studies suggest that E<sub>2</sub> can preferentially stimulate the proliferation of prostate stromal cells.

This is the first report on oestrogen metabolism by 17 $\beta$ HSD in prostatic fibroblasts and epithelial cells in primary culture. Both cell types exhibited 17 $\beta$ HSD activity converting E<sub>2</sub> to E<sub>1</sub> but not vice versa. The rates of conversion of E<sub>2</sub> to E<sub>1</sub> in epithelial cells (11.89 pmol/mg protein/h) and fibroblasts (6.12 pmol/mg protein/h) in the present study are comparable to that of testosterone oxidation to androstenedione in prostatic fibroblasts in primary culture (3.1 pmol/mg protein/h, Tsugaya et al. 1996). This observation of 17 $\beta$ HSD metabolism of E<sub>2</sub> but not E<sub>1</sub> in primary cultures is in keeping with previous reports on the expression of 17 $\beta$ HSD types 2, 4 and 5 (but not type 1) in the human prostate tissue (reviewed in section 1.5). Whereas 17 $\beta$ HSD types 2 and 4 exhibit mainly dehydrogenase activity and can oxidise E<sub>2</sub> to E<sub>1</sub>, type 1 17 $\beta$ HSD catalyses the reduction of E<sub>1</sub> to E<sub>2</sub> and type 5 17 $\beta$ HSD the conversion of androstenedione to testosterone (Peltoketo et al. 1999, Labrie et al. 2000). Therefore, it is likely that 17 $\beta$ HSD metabolism of oestrogens occurs mainly in the oxidative direction converting E<sub>2</sub> to the weaker oestrogen, E<sub>1</sub>, not only in prostatic cells *in vitro* but also in prostate tissue *in vivo*. However, it should be borne in mind that any measurement of 17 $\beta$ HSD activity is the sum of

both reductive and oxidative reactions catalysed by the various types of 17 $\beta$ HSD expressed at different levels in a system. The lack of conversion of E<sub>1</sub> to E<sub>2</sub> in prostatic cells does not necessarily equate to the absence of enzymes capable of catalysing E<sub>1</sub> reduction but can simply be due to relatively high activities of 17 $\beta$ HSD enzymes converting E<sub>2</sub> to E<sub>1</sub>, with the net result of E<sub>2</sub> oxidation in these cells.

## **Chapter 5**

### **Results:**

#### ***Effects of Permixon® on Oestrogen Metabolism in Prostatic Cells in Primary Culture***

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- 5.1 Effects of Permixon® on interaction between 5 $\alpha$ R1 and aromatase
- 5.2 Effects of Permixon® on oestradiol catabolism in prostatic cells in primary culture
- 5.3 Discussion

Although Permixon<sup>®</sup> is commercially available as a phytotherapeutic agent for the management of BPH, its precise mechanism of action is unknown. Besides inhibiting the activity of both 5 $\alpha$ -reductase isozymes in vitro (Iehle et al. 1995, Bayne et al. 1999), it has also been shown to decrease the nuclear concentrations of oestrogen receptor in BPH prostate tissue (Di Silverio et al. 1992). Indeed, oestrogens have long been implicated in the pathogenesis of BPH (reviewed in section 1.7.3a) and their concentrations in the prostate may be regulated by various oestrogen-metabolising enzymes. To better understand the mechanism of action of Permixon<sup>®</sup>, this chapter aims at investigating the effects of Permixon<sup>®</sup> on oestrogen anabolism (aromatase activity) and catabolism (17 $\beta$ HSD activity) in prostatic cells in primary culture.

## **5.1 Effects of Permixon<sup>®</sup> on Interaction between 5 $\alpha$ R1 and Aromatase**

Results from Chapter 3 reveal that in cells expressing both 5 $\alpha$ R1 and aromatase, inhibition of 5 $\alpha$ R1 led to an increase in aromatase activity but not vice versa. Previous studies have shown that Permixon<sup>®</sup> inhibited 5 $\alpha$ R1 activity in a baculovirus-directed expression system (Iehle et al. 1995) and in human prostatic cells in primary culture (Bayne et al. 1999). Further experiments were therefore carried out to examine if Permixon<sup>®</sup>, a known 5 $\alpha$ R1-inhibiting agent, would also affect the regulatory interaction between 5 $\alpha$ R1 and aromatase, first in a co-transfection model and then in prostatic cells in primary culture.

### **5.1.1 Effects of Permixon<sup>®</sup> on COS-1 cells co-transfected with plasmids encoding 5 $\alpha$ R1 and aromatase**

To investigate any effect of Permixon<sup>®</sup> on the interaction between 5 $\alpha$ R1 and aromatase, the co-transfection model described in sections 3.2 & 3.3 was used.

Briefly, COS-1 cells co-transfected with plasmids encoding human 5 $\alpha$ R1 (pCMV2.5 $\alpha$ R1) and aromatase (pCMV2.arom) were incubated with 10 nM androstenedione and various concentrations of Permixon<sup>®</sup> for 3 h. Steroids were then extracted from the medium and analysed by HPLC; cells were lysed and luciferase activity measured as described in sections 2.6.1 & 2.5.4 respectively. Initially, concentrations of Permixon<sup>®</sup> up to 10 mg/l were used as described in previous studies, which reported significant inhibition of 5 $\alpha$ R1 activity by 10 mg/l Permixon<sup>®</sup> in prostatic cells in primary culture (Bayne et al. 1999). In the present study, however, 10 mg/l Permixon<sup>®</sup> did not affect 5 $\alpha$ R1 activity expressed in COS-1 cells transfected with either pCMV2.5 $\alpha$ R1 only or both pCMV2.5 $\alpha$ R1 and pCMV2.arom (data not shown). The concentration of Permixon<sup>®</sup> was therefore increased to 200 mg/l in subsequent experiments.

Permixon<sup>®</sup> (up to 200 mg/l) did not affect aromatase activity in cells transfected with pCMV2.arom only (fig. 5.1a), whereas 5 $\alpha$ R1 activity in cells transfected with pCMV2.5 $\alpha$ R1 only was inhibited by 100-200 mg/l Permixon<sup>®</sup> (fig. 5.1b). In COS-1 cells co-transfected with both pCMV2.5 $\alpha$ R1 and pCMV2.arom, Permixon<sup>®</sup> not only inhibited 5 $\alpha$ R1 activity but also led to a concomitant increase in aromatase activity (fig. 5.1c). This effect of Permixon<sup>®</sup> on the regulatory interaction between the two enzymes is similar to that of the 5 $\alpha$ R1-specific inhibitor LY306089 described in section 3.3.2 (fig. 3.6, pp.98-99).

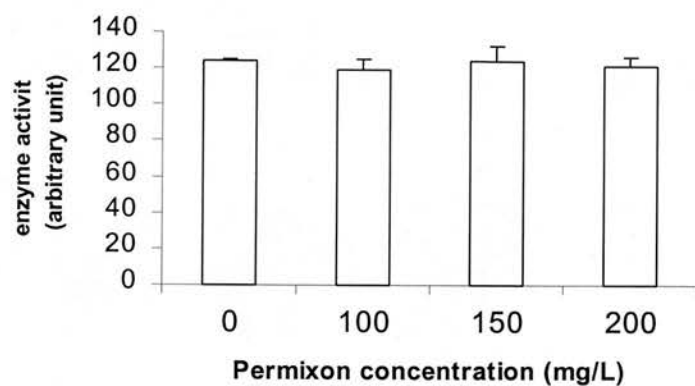
#### 5.1.2 Effects of Permixon<sup>®</sup> on the interaction between 5 $\alpha$ R1 and aromatase in prostatic cells in primary culture

Previous experiments (Bayne et al. 1999) and preliminary work at the beginning of this project (data not shown) demonstrated that Permixon<sup>®</sup> at a concentration of 20 mg/l or above (but not 10 mg/l) inhibited the proliferation of both epithelial cells and fibroblasts in primary culture. To investigate the effects of Permixon<sup>®</sup> on the interaction between 5 $\alpha$ R1 and aromatase in prostatic cells in primary culture, the concentrations of Permixon<sup>®</sup> used in medium were thus limited to 10 mg/l or below.

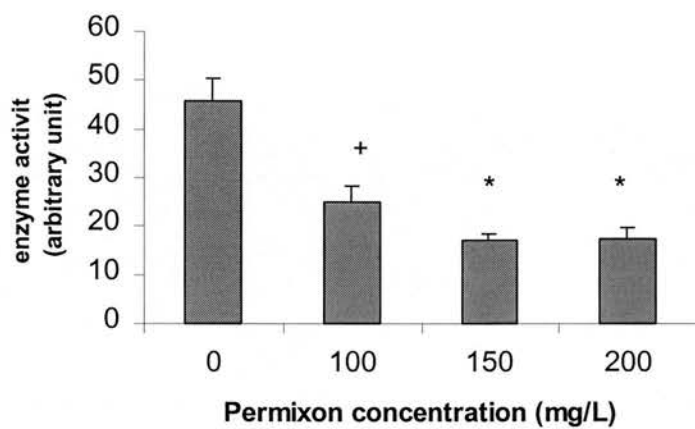
**Figure 5.1 Effects of Permixon<sup>®</sup> on enzyme activities in COS-1 cells transfected with (a) pCMV2.arom (aromatase-encoding plasmid) only, (b) pCMV2.5 $\alpha$ R1 (5 $\alpha$ R1-encoding plasmid) only, and (c) both pCMV2.arom and pCMV2.5 $\alpha$ R1.** Enzyme activities are expressed as mean of triplicates  $\pm$  s.e.m. Arbitrary unit of steroid-metabolising enzyme activity is calculated by the following formula: steroid formation (pmol)  $\times 10^6$  / luciferase activity (relative light unit). Results are representative of 3 separate experiments. <sup>+</sup>P<0.05 and \*P<0.01 compared with controls. Open bars, aromatase; solid bars, 5 $\alpha$ R1.



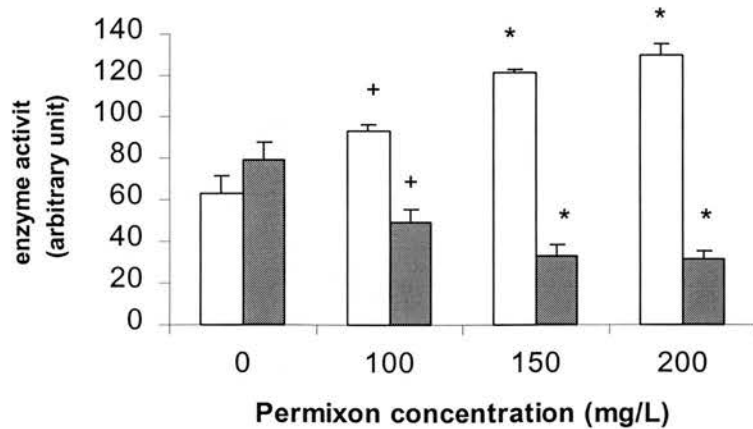
**(a) Aromatase only**



**(b) 5 $\alpha$ R1 only**



**(c) Both aromatase & 5 $\alpha$ R1**



In section 3.5, epithelial cells in primary culture converted androstenedione to both 5 $\alpha$ A (5 $\alpha$ R1 activity) and testosterone (17 $\beta$ HSD activity), but not E<sub>1</sub> (aromatase activity). Here Permixon<sup>®</sup> (10 mg/l) was shown to inhibit 5 $\alpha$ R1 activity in epithelial cells in primary culture as predicted (fig. 5.2a), whereas metabolism of androstenedione to testosterone was not affected by up to 10 mg/l Permixon<sup>®</sup> (fig. 5.2b). No E<sub>1</sub> or E<sub>2</sub> was detectable even in the presence of 10 mg/l Permixon<sup>®</sup>.

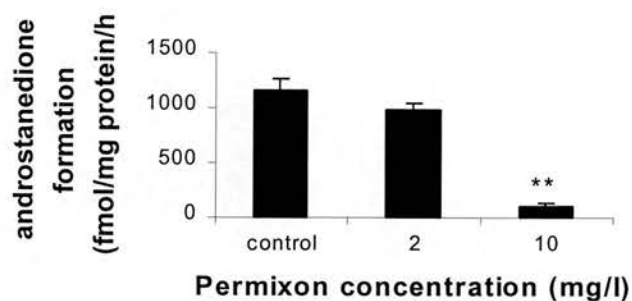
In contrast, androstenedione was converted to 5 $\alpha$ A, E<sub>1</sub> and testosterone in prostatic fibroblasts in primary culture. Permixon<sup>®</sup> (10 mg/l) decreased 5 $\alpha$ A formation in fibroblasts as expected (fig. 5.3a), whereas the metabolism of androstenedione to testosterone was unaffected by Permixon<sup>®</sup> up to 10 mg/l (fig. 5.3b). In fibroblasts in primary culture, Permixon<sup>®</sup> increased the conversion of androstenedione to E<sub>1</sub> in a dose-dependent manner (fig. 5.3c). Again, the effects of Permixon<sup>®</sup> on 5 $\alpha$ A and E<sub>1</sub> formation in prostatic fibroblasts are similar to those of the 5 $\alpha$ R1 specific inhibitor LY306089.

## **5.2 Effects of Permixon<sup>®</sup> on Oestradiol Catabolism in Prostatic Cells in Primary Culture**

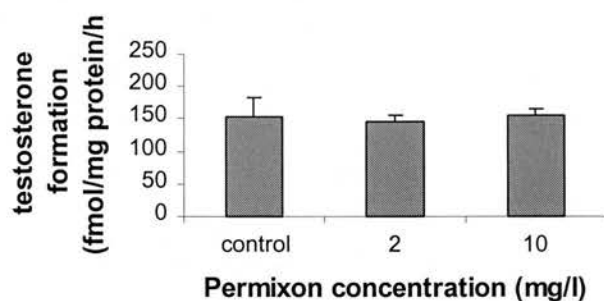
The above data demonstrate that by inhibiting 5 $\alpha$ R1, Permixon<sup>®</sup> can indirectly increase aromatase activity and hence local oestrogen formation from androgen precursors (oestrogen anabolism) in prostatic cells. In Chapter 4, prostatic fibroblasts and epithelial cells in primary culture were also shown to actively metabolise E<sub>2</sub> to the weaker oestrogen E<sub>1</sub>; no conversion from E<sub>1</sub> to E<sub>2</sub> was detectable in either cell type. The next set of experiments aimed to examine if Permixon<sup>®</sup> can also affect E<sub>2</sub> catabolism (17 $\beta$ HSD activity) in prostatic cells.

Epithelial cells and fibroblasts in primary culture were incubated with radiolabelled E<sub>2</sub> and various concentrations of the phytotherapeutic agent. To avoid any effect of Permixon<sup>®</sup> on cell proliferation, concentrations of Permixon<sup>®</sup> used were again limited to 10 mg/l or less. Preliminary experiments of E<sub>2</sub> catabolism (17 $\beta$ HSD activity) in both cell types have been described in section 4.4.

(a)  $A \rightarrow 5\alpha A$



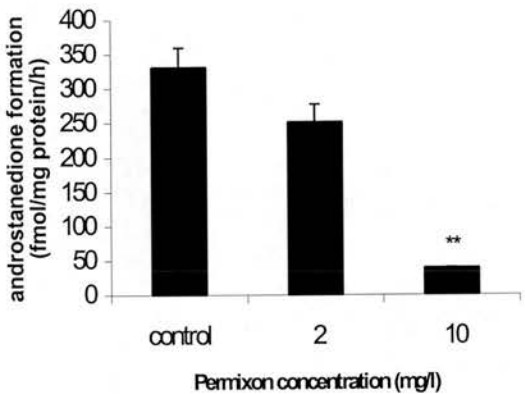
(b)  $A \rightarrow T$



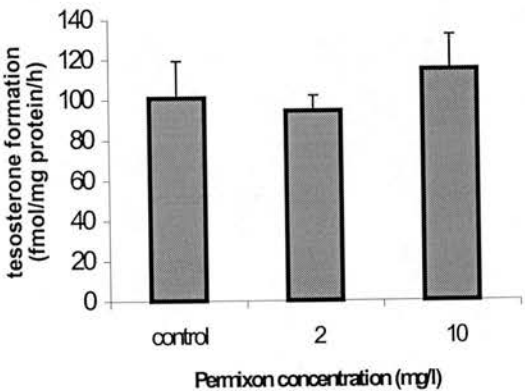
**Fig. 5.2** Effects of Permixon<sup>®</sup> on the metabolism of androstenedione to (a)  $5\alpha$ -androstenedione, and (b) testosterone in epithelial cells in primary culture. Values are mean of triplicates  $\pm$  s.e.m. \*\* $P < 0.001$ , compared with controls. A, androstenedione;  $5\alpha A$ ,  $5\alpha$ -androstenedione; T, testosterone. Results are representative of 2 experiments using prostatic epithelial cells cultured from 2 different BPH patients. For experimental details, please refer to fig. 3.13 (pp. 112-113). No aromatase activity (formation of  $E_1$  or  $E_2$ ) was detectable in epithelial cells.

**Fig. 5.3 Effects of Permixon<sup>®</sup> on the metabolism of androstenedione to (a) 5 $\alpha$ -androstanedione, (b) testosterone, and (c) oestrone in prostatic fibroblasts in primary culture.** Values are mean of triplicates  $\pm$  s.e.m. \*\*P<0.001, <sup>+</sup>P<0.05 compared with controls. A, androstenedione; 5 $\alpha$ A, 5 $\alpha$ -androstanedione; E<sub>1</sub>, oestrone; T, testosterone. For experimental details, please refer to fig. 3.12 (pp. 110-111). Results are representative of 3 separate experiments using prostatic fibroblasts cultured from 3 different BPH patients.

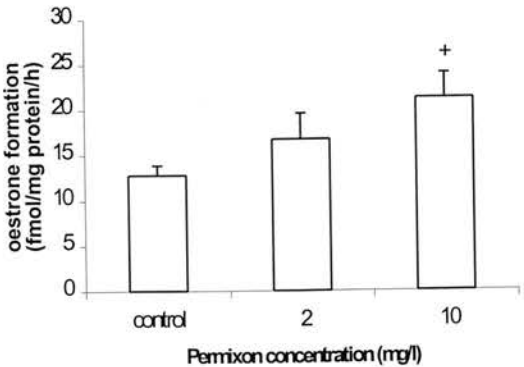
(a)  $A \rightarrow 5\alpha A$



(b)  $A \rightarrow T$



(c)  $A \rightarrow E_1$

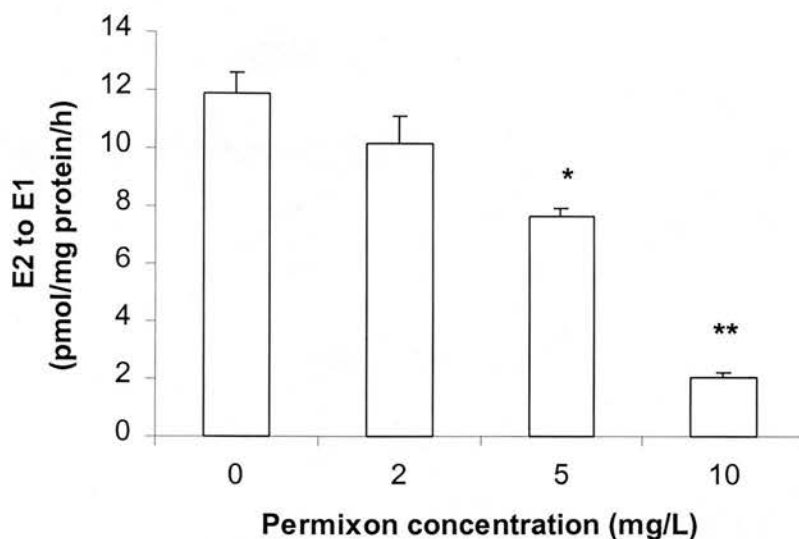


In epithelial cells in primary culture, Permixon<sup>®</sup> inhibited the conversion of E<sub>2</sub> to E<sub>1</sub> in a dose-dependent manner (IC<sub>50</sub> = 7.8 mg/l, fig. 5.4). In comparison, 10 mg/l Permixon<sup>®</sup> decreased E<sub>2</sub> metabolism to E<sub>1</sub> by approximately 18% in fibroblasts (fig. 5.5).

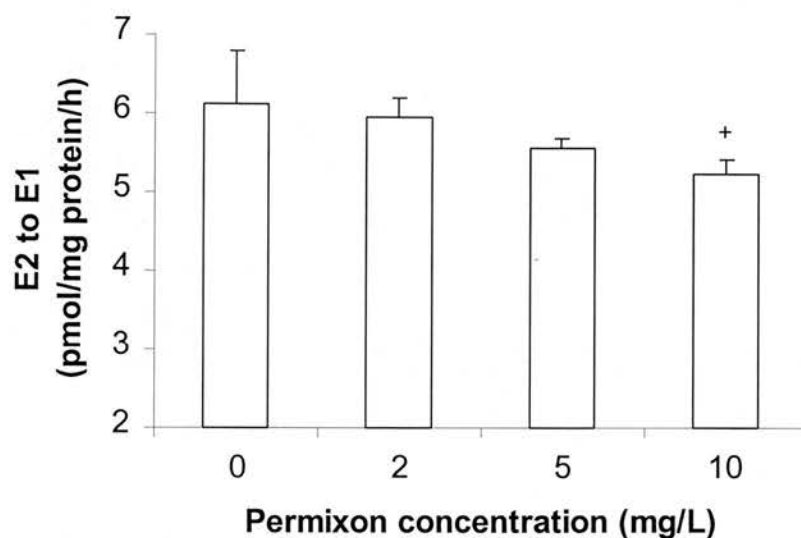
### 5.3 Discussion

Intraprostatic oestrogen concentrations depend, among other factors, on the availability of androgen precursors and local expression of oestrogen-producing and oestrogen-inactivating enzymes. In this chapter, Permixon<sup>®</sup> is demonstrated to have two effects on oestrogen metabolism in prostatic cells in primary culture. Firstly, Permixon<sup>®</sup> indirectly increased aromatase activity and thus local oestrogen biosynthesis by inhibiting 5 $\alpha$ R1 in fibroblasts. Secondly, by inhibiting the conversion of E<sub>2</sub> to E<sub>1</sub>, it increased the concentrations of the more active oestrogen in both epithelial cells and fibroblasts. This is the first report on the effects of Permixon<sup>®</sup> on oestrogen metabolism in prostatic cells.

These pro-oestrogenic properties of Permixon<sup>®</sup> are in contrast to the only previous study reporting on the phytotherapeutic agent's interference with oestrogen action, in which Permixon<sup>®</sup>-treated patients were shown to have decreased concentrations of nuclear, but not cytosolic, oestrogen receptors in their prostate tissue compared with the placebo-treated group (Di Silverio et al. 1992). In the same study, nuclear concentrations of both androgen and progesterone receptors were also lower in the Permixon<sup>®</sup>-treated group than the control group; total concentrations of oestrogen, androgen and progesterone receptors in the prostate were however not mentioned in the study. The decreased concentrations of nuclear oestrogen, androgen and progesterone receptors were most likely due to damage of nuclear membrane caused by Permixon<sup>®</sup> demonstrated in a later study (Bayne et al. 2000).



**Fig. 5.4 Effects of Permixon<sup>®</sup> on the conversion of oestradiol (E<sub>2</sub>) to oestrone (E<sub>1</sub>) in epithelial cells in primary culture.**  $5 \times 10^4$  cells/well in triplicate were plated in 6-well plates in serum-free EGM. 48 h later, the medium was replaced by 2 ml serum-free EGM containing 10 nM radiolabelled E<sub>2</sub> and various concentrations of Permixon<sup>®</sup> or ethanol in control wells (1%, v/v). The medium was collected after 4 h incubation. Steroids were extracted with ethyl acetate and analysed by HPLC. Cells were lysed and protein concentrations were determined as described in sections 2.6.2 & 2.3.4 respectively. Results are expressed as mean  $\pm$  s.e.m. (n=9) from 3 separate experiments using epithelial cells (passage number 1) from 3 BPH patients. \* $P < 0.01$ , \*\* $P < 0.001$ , compared with controls.



**Fig. 5.5 Effects of Permixon<sup>®</sup> on the conversion of oestradiol (E<sub>2</sub>) to oestrone (E<sub>1</sub>) in prostatic fibroblasts in primary culture.**  $5 \times 10^4$  cells/well in triplicate were plated in 6-well plates in phenol red-free RPMI supplemented with 10% DCC-FCS. 48 h later, the medium was replaced by 2 ml of the same medium containing 10 nM radiolabelled E<sub>2</sub> and various concentrations of Permixon<sup>®</sup> or 1% ethanol (v/v) in control wells. The medium was collected after 4 h incubation. Steroids were extracted with ethyl acetate and analysed by HPLC. Cells were lysed and protein concentrations were determined as described in sections 2.6.2 & 2.3.4 respectively. Results are expressed as mean  $\pm$  s.e.m. (n=9) from 3 separate experiments using fibroblasts (passage number 2 or 3) from 3 BPH patients. <sup>+</sup> $P < 0.05$ , compared with controls.



Using electron microscopy, Bayne et al. (2000) demonstrated widespread damage to the nuclear membrane in prostatic epithelial cells and fibroblasts in primary culture treated with 10 mg/l Permixon<sup>®</sup> for 2 d. It is therefore conceivable that the steroid receptors in Permixon<sup>®</sup>-treated prostates in the previous study by Di Silverio et al. (1992) either diffused through damaged nuclear membrane from the nucleus to the cytosol or failed to be transported from the cytosolic to the nuclear compartment. While classical oestrogen action depends on the presence of oestrogen receptor-ligand complex within the nucleus, it is not clear if the phytotherapeutic agent's effects on local oestrogen concentrations would be counteracted by its effects on nuclear receptor concentrations.

In this study, higher concentrations of Permixon<sup>®</sup> were required to inhibit 5 $\alpha$ -reductase activity in COS-1 cells than in prostatic cells in primary culture. This is in keeping with a previous report in which widespread morphological changes were observed in prostatic cells in primary culture treated with Permixon<sup>®</sup> (10 mg/l) but not in other cell cultures including epididymal, testicular, kidney, skin and breast cells (Bayne et al. 2000). Moreover, after oral administration, ingredients of Permixon<sup>®</sup> including lauric acid, oleic acid and  $\beta$ -sitosterol were selectively concentrated in the rat prostate to the exclusion of other organs (Chevalier et al. 1997). Given that fatty acids, essential components of cell and nuclear membranes, constitute approximately 90% of all ingredients of Permixon<sup>®</sup>, the cell-type selectivity of the phytotherapeutic agent might possibly be explained by differences in phospholipid and fatty acid composition between different cell types.

## ***Chapter 6***

### ***Final Discussion and Future Studies***

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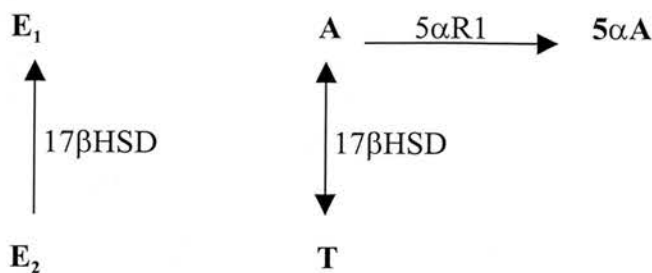
The present study was designed to examine, on the one hand, the role of 5 $\alpha$ -reductase type 1 isozyme in the human prostate, and on the other hand, the effects of oestrogens on prostatic cells. Two hypotheses are tested in this thesis. Firstly, the hypothesis that 5 $\alpha$ R1 can regulate aromatase activity was investigated initially in a co-transfection model and then in primary cultures of prostatic cells. Secondly, it was hypothesised that prostatic cells are not only oestrogen target cells expressing oestrogen receptors but also capable of further metabolism of oestrogens, thereby modulating their effects on the prostate. Furthermore, the mechanism of action of Permixon<sup>®</sup>, a phytotherapeutic agent for the treatment of BPH, is not entirely clear; the effects of Permixon<sup>®</sup> on oestrogen metabolism in primary cultures of prostatic cells were also explored.

### ***Steroid metabolism in primary cultures of prostatic cells***

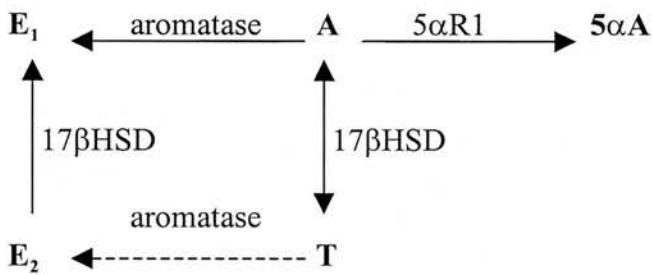
Steroid metabolic pathways examined in this thesis are summarised in fig. 6.1. In epithelial cells in primary culture, androstenedione was converted to both 5 $\alpha$ A (by 5 $\alpha$ R1) and testosterone (by 17 $\beta$ HSD). Testosterone and androstenedione were interconverted to each other in epithelial cells. E<sub>2</sub> was metabolised to E<sub>1</sub> but not vice versa (fig. 6.1a). Aromatase activity was not detectable in epithelial cells.

With the exception of the aromatase activity expression, steroid metabolic pathways found in fibroblasts are similar to those in epithelial cells (fig. 6.1b). Androstenedione was metabolised to E<sub>1</sub> (by aromatase), 5 $\alpha$ A (by 5 $\alpha$ R1) and testosterone (by 17 $\beta$ HSD). 17 $\beta$ HSD also oxidised both testosterone to androstenedione and E<sub>2</sub> to E<sub>1</sub>; however, E<sub>1</sub> was not converted to E<sub>2</sub> in fibroblasts. Despite the presence of aromatase activity in fibroblasts, E<sub>2</sub> was not detectable as a metabolite when androstenedione was added to fibroblasts as a substrate. This was possibly due to the relatively high oxidative 17 $\beta$ HSD activity in fibroblasts removing any E<sub>2</sub> formed by testosterone aromatisation in the same cells. Therefore, in interpreting results of steroid metabolism in cell cultures, the highly dynamic and complex metabolic pathways that are potentially involved need to be taken into account.

**(a) Epithelial cells**



**(b) Fibroblasts**



**Figure 6.1 Overview of steroid metabolic pathways in prostatic cells in primary culture reported in the present study.**  $5\alpha$ -reductase type 1 isozyme ( $5\alpha\text{R1}$ ) activity is present in both (a) epithelial cells and (b) fibroblasts in primary culture, reducing androstenedione ( $A$ ) to  $5\alpha$ -androstenedione ( $5\alpha A$ ).  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta\text{HSD}$ ) activity occurs predominantly in the oxidative direction, converting oestradiol ( $E_2$ ) to oestrone ( $E_1$ ) and testosterone ( $T$ ) to  $A$ .  $17\beta\text{HSD}$  reduction of  $A$  to  $T$  is also present in both cells types. Aromatase activity occurs in fibroblasts in primary culture, converting  $A$  to  $E_1$ , but not in epithelial cells. Aromatisation of  $T$  to  $E_2$  (broken arrow) has not been demonstrated in the present thesis but possibly occurs in fibroblasts in primary culture.

### *Interaction between 5 $\alpha$ R1 and aromatase*

In both the co-transfection model and fibroblasts in primary culture, inhibition of 5 $\alpha$ R1 activity by either LY306086 or Permixon<sup>®</sup> led to an increase in aromatase activity. By converting androstenedione to a non-aromatisable steroid, 5 $\alpha$ R1 can limit substrate availability to the enzyme aromatase. An alternative explanation for the observed regulatory interaction between the two enzymes is that 5 $\alpha$ A, the product of 5 $\alpha$ R1 metabolism of androstenedione, acts as an inhibitor of aromatase. 5 $\alpha$ A was indeed reported to be an inhibitor of aromatase activity in human breast carcinoma cells (Perel et al. 1984) and in rat granulosa cells (Hillier et al. 1980). The aromatisation of androstenedione (4 nM) in human breast carcinoma cells in primary culture was inhibited approximately 70% and 95% by 10 nM and 100 nM 5 $\alpha$ A respectively (Perel et al. 1984). The same study also reported that breast carcinoma cells with the most active formation of 5 $\alpha$ -reduced androgen metabolites including 5 $\alpha$ A had the lowest rate of androstenedione aromatisation, whereas those cells with the lowest formation of 5 $\alpha$ -reduced metabolites had the highest aromatase activities. Likewise, in another study, Hillier et al. (1980) reported that in rat granulosa cells, testosterone aromatisation was competitively inhibited by 5 $\alpha$ A; 1  $\mu$ M 5 $\alpha$ A decreased the aromatisation of 100 nM by 73%. In both of these studies, other 5 $\alpha$ -reduced steroids were found to be less potent aromatase inhibitors than 5 $\alpha$ A; these steroids were dihydrotestosterone (DHT), androsterone, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ Adiol) and 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ Adiol) (Perel et al. 1984, Hillier et al. 1980). It is thus possible that by converting androstenedione to 5 $\alpha$ A, 5 $\alpha$ R1 not only limits aromatase access to its substrate, but also produces a steroid metabolite which can directly inhibit the enzyme aromatase. Further kinetic studies are required to examine the effects of 5 $\alpha$ A and other 5 $\alpha$ -reduced androgen metabolites on aromatase activity in prostatic cells.

Although inhibition of 5 $\alpha$ R1 by either LY306089 or Permixon<sup>®</sup> led to an increase of aromatase activity in fibroblasts, the conversion of androstenedione to

testosterone (17 $\beta$ HSD activity) was unaffected by 5 $\alpha$ R1 inhibition in either epithelial cells or fibroblasts in primary culture. One possible explanation is that 5 $\alpha$ A is not an inhibitor of 17 $\beta$ HSD conversion of androstenedione to testosterone in either cell type. Alternatively, though 5 $\alpha$ R1 inhibition led to increased androstenedione availability for 17 $\beta$ HSD reduction to testosterone, very high oxidative 17 $\beta$ HSD activity present in the same cells might have prevented the increased formation of testosterone from androstenedione.

Aromatase mRNA and activity were found in fibroblasts but not in epithelial cells in primary culture in the present study. Yet 5 $\alpha$ R1 mRNA levels (Habib et al. 1998) and activity in epithelial cells (present study, section 3.5) were shown to be higher than in stromal cells. The function of 5 $\alpha$ R1 in epithelial cells awaits further elucidation. A speculative role for 5 $\alpha$ R1 in epithelial cells is that it may, in conjunction with various 17 $\beta$ HSD isozymes and 5 $\alpha$ R2, regulate androgen activation and inactivation, and thus play a part in the control of androgen response in the prostate.

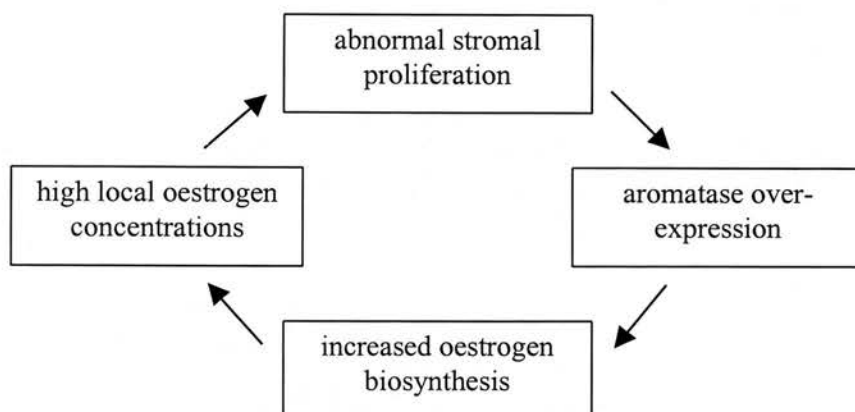
### ***Balance between 5 $\alpha$ R1 and 5 $\alpha$ R2***

In considering a potential role for 5 $\alpha$ R1 in androstenedione catabolism, it should be noted that 5 $\alpha$ R2 is in general regarded as the more important of the two isozymes within the human prostate. As well as being the predominant isozyme, it also exhibits higher substrate affinity than 5 $\alpha$ R1 (Thigpen et al. 1993a, Levy et al. 1995). Although the role of 5 $\alpha$ R1 may be minor compared with that of 5 $\alpha$ R2, it is feasible that a balance in the expression of the two isozymes is important to the normal control of intra-prostatic androgen/oestrogen balance. Evidence suggests that increased 5 $\alpha$ R1 or decreased 5 $\alpha$ R2 expression may be associated with malignancy of the human prostate. Firstly, recent data show that 5 $\alpha$ R1 mRNA expression was higher in prostate cancer than in normal or hyperplastic tissues, but no difference in 5 $\alpha$ R2 mRNA levels was found between normal and malignant tissues (Iehle et al. 1999). Secondly, the catalytic efficiency ( $V_{\max}/K_m$ ) of 5 $\alpha$ R1 in stromal tissues of

malignant tissue was 3 times higher than in BPH (Hudson & Wherrett 1990). Thirdly, in contrast to normal prostate and BPH, where  $5\alpha R2$  is the major isozyme, in prostate cancer cell lines including DU145, LNCaP and PC3,  $5\alpha R1$  is the predominant  $5\alpha$ -reductase isozyme (Smith et al. 1996, Negri-Cesi et al. 1998, Negri-Cesi et al. 1999). Whether malignant prostate tissues have increased  $5\alpha R1$  expression compared with normal tissues, and thus decreased local oestrogen biosynthesis, is unknown. However, oestrogens have been shown to directly inhibit the growth of prostatic adenocarcinoma in rat (Daehlin et al. 1987) and the proliferation of PC3 cells, a human prostate cancer cell line (Lau et al. 2000). If increased  $5\alpha R1$  expression or imbalance between the two  $5\alpha$ -reductase isozymes is a contributing factor to malignancy,  $5\alpha R1$  may open new doors to the treatment and prevention of prostatic carcinoma.

### *Aromatase*

In this study, both aromatase mRNA and activity were detectable in primary cultures of fibroblasts but not in epithelial cells. Similarly, in both BPH and normal prostate tissue sections, aromatase immunostaining in the epithelium was either absent or much lower than in the stroma (Matzkin & Soloway 1992, Hiramatsu et al. 1997). It is therefore very likely that in vivo aromatase activity is higher in the stroma than in the epithelium of prostatic tissue. In a study comparing benign and malignant conditions of the prostate, Schweikert (1979) reported higher aromatase activity in fibroblasts cultured from BPH than those cultured from cancer specimens. As a result, oestrogens locally produced by the high aromatase activities in BPH fibroblasts could thus be a contributing factor to the pathogenesis of BPH. This is consistent with the finding that  $E_2$  stimulated the proliferation of fibroblasts in primary culture (section 4.2). Hence aromatase may play a potential role in the pathogenesis of BPH as suggested in figure 6.2.



**Fig. 6.2 Schematic diagram outlining a hypothesised role for aromatase in BPH pathogenesis.** Here it is proposed that a yet unknown mechanism can trigger abnormal stromal proliferation and thus an overall increase in aromatase activity, which in turn produces more oestrogens locally, stimulating the proliferation of more stromal cells. The increasing plasma oestrogen/androgen ratio with advancing age is a possible factor in triggering abnormal proliferation of prostatic stromal cells.



Aromatase expression in epithelial cells in primary culture was undetectable (present study) and aromatase immunostaining in the epithelium in both BPH and normal prostate tissue sections was either absent or much lower than in the stroma (Matzkin & Soloway 1992, Hiramatsu et al. 1997). Yet mRNA of both oestrogen receptor subtypes have been identified in epithelial cells in primary culture (present study) and immunostaining of ER $\alpha$  and ER $\beta$  was reported in the epithelial compartment of human prostate tissues (Royuela et al. 2001, Pasquali et al. 2001a). A speculative explanation is that oestrogens locally produced by aromatase activity in the stroma/fibroblasts act in a paracrine manner on neighbouring epithelial cells. Whether a stromal-epithelial interaction via oestrogens is important to normal or pathological prostate development needs further investigation. Also, the effects of oestrogens on prostatic epithelial cells are not entirely clear but this area of research will be highly relevant to the understanding and treatment of prostate cancer, which is an epithelial disease. Further studies to examine the effects of oestrogens on epithelial cells in primary culture could compare (both qualitatively and quantitatively) the protein expression patterns in cells treated with various oestrogenic compounds with those in control cells.

### ***Oestrogens***

In considering a role for oestrogens in BPH pathogenesis, it is important to note that oestrogens can exert both direct and indirect effects on the prostate. Firstly, systemic administration of oestrogens can inhibit gonadotrophin secretion, leading to decreased testicular output of androgens and lowered plasma androgen levels, which in turn cause regressive changes in the prostate. Secondly, oestrogens, either derived from plasma or produced locally within the prostate, can exert their effects directly on prostatic cells. It is likely that oestrogen response in the prostate depends on a number of factors including plasma levels of oestrogens, local concentrations of oestrogens, expression of oestrogen receptor subtypes, interaction between oestrogen receptors and co-activators/co-repressors and so on.

It is paradoxical that BPH, an androgen-dependent disease, manifests in men at a time when their testicular activities and plasma androgen levels are declining. However, with advancing age, plasma oestrogen/androgen ratios are increased (reviewed in section 1.7.3a). Results from in vitro fibroblast proliferation experiments in the present study and from previous in vivo experiments in dog (Walsh & Wilson 1976, DeKlerk et al. 1979) strongly suggest a role for oestrogens in BPH pathogenesis. However, whether increased plasma oestrogen/androgen ratios or increased local oestrogen concentrations due to oestrogen biosynthesis are responsible for BPH is currently unknown. Sharpe (2001) suggested that a balance in action between androgens and oestrogens may be a critical factor in the normal development of the male reproductive system; exposure of the fetal testis to an abnormal endocrine environment (decreased ratios of androgens to oestrogens) may lead to testicular cancer, cryptorchidism, hypospadias and decreased sperm counts. Whether BPH is related to this group of testicular dysgenesis disorders requires further investigations.

Besides  $E_2$ ,  $3\beta$ Adiol was also shown to stimulate the proliferation of fibroblasts in primary culture.  $3\beta$ Adiol binds to both  $ER\alpha$  and  $ER\beta$  (Kuiper et al. 1997) but not androgen receptor (Turcotte et al. 1988). Although compared with  $E_2$ , the relative binding affinities of  $3\beta$ Adiol for  $ER\alpha$  and  $ER\beta$  were only 3% and 7% respectively (Kuiper et al. 1997),  $3\beta$ Adiol concentrations are 10 times higher than those of  $E_2$  in plasma and >30 times higher in BPH prostate tissues (reviewed in section 1.7.3a). Recently, Weihua et al. (2001) suggested that  $3\beta$ Adiol may be an important endogenous  $ER\beta$  ligand in the prostate; it decreased prostatic androgen receptor levels in wild-type but not  $ER\beta$  knockout mice. Data from the present study show that both  $3\beta$ Adiol and  $E_2$  stimulated proliferation of prostatic fibroblasts to a similar extent. Moreover, maximum stimulation occurred at 100 nM for both compounds in medium supplemented with 2% DCC-FCS, indicating that  $3\beta$ Adiol may also have significant oestrogenic effects in vivo. Having higher affinity for  $ER\beta$  than  $ER\alpha$  (Kuiper et al. 1997),  $3\beta$ Adiol can potentially elicit different effects to  $E_2$ , which has similar affinity for both ER subtypes.

Another putative oestrogen which may be of importance in prostatic pathophysiology is androst-5-ene-3 $\beta$ ,17 $\beta$ -diol (A5diol). It can be synthesised from dehydroepiandrosterone, the most abundant steroid in plasma, by the action of 17 $\beta$ HSD, and further metabolised by 3 $\beta$ HSD to testosterone. Compared with E<sub>2</sub>, its relative binding affinities to ER $\alpha$  (6%) and ER $\beta$  (17%) are even higher than those of 3 $\beta$ Adiol (Kuiper et al. 1997). Also, concentrations of A5diol in both plasma and BPH tissues are higher than those of 3 $\beta$ Adiol and E<sub>2</sub> (table 1.7, p.36). If 3 $\beta$ Adiol and A5diol are proven to be more important oestrogens than E<sub>2</sub> in the human prostate, our understanding of the balance and interconversion between androgens and oestrogens within the gland will need to be re-examined.

To date, the only comprehensive study reporting the binding affinities of various oestrogenic compounds to the two ER subtypes compared the human ER $\alpha$  with the rat ER $\beta$  (Kuiper et al. 1997). Given that findings in one species cannot always be extrapolated to another, it would be useful to know the binding affinities of various oestrogens, including E<sub>2</sub>, 3 $\beta$ Adiol and A5diol, to the human ER $\beta$ . Yet binding affinity of a compound to ER is not equivalent to its potential in eliciting oestrogen action. To better understand the role of the putative oestrogens 3 $\beta$ Adiol and A5diol in oestrogen action in the prostate, their abilities to transactivate oestrogen-responsive genes can also be examined. To this end, cells can be transfected with plasmids encoding either ER subtype and a known oestrogen responsive gene linked to a reporter system. Addition of an oestrogenic compound to the transfected cells will then result in binding of the ligand to the ER subtype, ER dimerisation, binding of the ER-ligand complex to oestrogen response elements (ERE), initiation of gene transcription and expression of the reporter gene. Compared with binding affinity studies, this type of transfection experiments gives a better indication as to the potential of an oestrogenic compound in eliciting functional oestrogen action. The work presented in this thesis has established that prostatic cells in primary culture express mRNA of both ER subtypes and that proliferation of fibroblasts is responsive to oestrogen stimulation. BPH-derived primary cell cultures would thus serve as a model for future investigations of the mechanism and regulation of action of oestrogens in the prostate.

## ***Permixon***

Two pro-oestrogenic properties of Permixon® on prostatic cells in primary culture were demonstrated in Chapter 5. E<sub>2</sub> conversion to E<sub>1</sub> by 17βHSD in epithelial cells was more sensitive to Permixon® inhibition than in fibroblasts; 10 mg/l Permixon® inhibited 17βHSD oxidation of E<sub>2</sub> in epithelial cells and fibroblasts by 75% and 18% respectively. In comparison, Delos et al. (1995) reported that testosterone oxidation to androstenedione by 17βHSD was also inhibited by Permixon® more so in primary cultures of epithelial cells (IC<sub>50</sub> = 40 mg/l) than in fibroblasts (IC<sub>50</sub> = 200 mg/l). One possible reason to this difference in the degree of 17βHSD inhibition is that different isozymes of 17βHSD were expressed in the two different cell types, which are differentially inhibited by Permixon®. Furthermore, the observation that 10 mg/l Permixon® did not affect androstenedione reduction to testosterone by 17βHSD also lends support to Permixon®'s selectivity on 17βHSD isozyme inhibition. Considering that 17βHSD isozymes are important regulators of steroid activation and inactivation within prostatic cells, further studies can identify the various 17βHSD isozymes expressed in primary cultures, with a view to specific 17βHSD isozyme regulation and possible therapeutic intervention.

Another pro-oestrogenic property of Permixon® reported in this thesis is the indirect increase in aromatase activity secondary to inhibition of 5αR1. However, if 3βAdiol is really a more important oestrogen within the prostate gland, Permixon® may have anti-oestrogenic effects by inhibiting both 5αR1 and 5αR2 activities, thereby depleting the intraprostatic supply of androgen precursors for 3βAdiol formation. Similarly, the effects of Permixon® on 17βHSD conversion of dehydroepiandrosterone to A5diol should also be examined.

The implications of the above pro-oestrogenic properties of Permixon® are currently unclear. Despite these pro-oestrogenic properties and the fact that oestrogens increased proliferation of fibroblasts in primary culture, Permixon® was

shown by studies to effectively decrease the size of prostate in BPH patients. Clinical follow-up studies are required to determine Permixon<sup>®</sup>'s long-term effects on BPH patients and their prostates, which have not been reported. Permixon<sup>®</sup> also contains small amount of phytosterols including  $\beta$ -sitosterol, which is a phytoestrogen. Phytoestrogens (natural oestrogenic compounds derived from soya, fruits and vegetables) are generally believed to be protective against the development of prostate cancer and contribute to the geographical differences in the prevalence of prostate cancer among different parts of the world. The soya-derived phytoestrogen genistein, for example, has high binding affinity to ER $\beta$  (36% compared with E<sub>2</sub>, Kuiper et al. 1997). It would be interesting to examine not only the interaction between phytoestrogens and the two ER subtypes but also their effects on prostatic cells in primary culture.

### ***Primary cultures of prostatic cells***

Compared with immortalised cell lines with repeated passaging, BPH-derived epithelial cells and fibroblasts in primary culture are closer to prostatic cells in vivo in terms of cellular characteristics. However, primary cell culturing is known to alter some of the endocrinological attributes of prostatic cells. For example, the expression of both 5 $\alpha$ R2 and androgen receptor in epithelial cells and fibroblasts quickly disappears after a short period of primary culture. In the present study, it is not clear if the process or conditions of cell culture had altered the mRNA expression patterns of aromatase and ER subtypes in prostatic cells. Differences in conditions of primary cultures, primer design or RT-PCR conditions between the present and previous studies may be the reason for different mRNA levels reported. Also, it is important to note that the presence of mRNA does not necessarily mean the expression of the encoded functional protein. However, the stimulatory effects of oestrogens on the proliferation of prostatic fibroblasts demonstrated in Chapter 4, which were antagonised by the anti-oestrogen ICI 182780, strongly suggest that either or both ER subtypes were indeed expressed in this cell type.

Proliferation of fibroblasts grown in medium supplemented with 10% DCC-FCS was stimulated by micromolar concentrations of  $E_2$  and  $3\beta$ Adiol, whereas nanomolar concentrations of both compounds were enough to increase the proliferation of fibroblasts in medium supplemented with 2% DCC-FCS. A speculative explanation is that 10% DCC-FCS stimulated the proliferation of fibroblasts to such an extent that high concentrations of oestrogens were required to further stimulate their growth. Alternatively, high DCC-FCS concentrations might have anti-oestrogenic effects on fibroblasts, e.g. down-regulation of ER gene expression. This difference in the oestrogen concentrations required to stimulate proliferation of fibroblasts grown in medium supplemented with different concentrations of DCC-FCS again highlights the fact that cell growth and characteristics are determined to a large extent by the culture conditions.

Lastly, despite the aforementioned shortcomings, prostatic cells in primary culture still offer advantages as a model in the study of steroid metabolism and actions in prostate. For instance, the results of ER mRNA expression in the two cell types and also the exclusive aromatase activity in fibroblasts are similar to immunohistochemical findings in prostatic tissues. The highly dynamic metabolic pathways reported in the present study may not be identical to those found in human prostatic cells *in vivo*; prostatic cells in primary culture remain possibly the model most representative of the *in vivo* situation to date.



## REFERENCES

- Ackerman GE, Smith ME, Mendelson CR, MacDonald PC, Simpson ER. 1981 Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. *J Clin Endocrinol Metab* 53:412-7.
- Andersson S, Russell DW. 1990 Structural and biochemical properties of cloned and expressed human and rat steroid 5 $\alpha$ -reductases. *Proc Natl Acad Sci USA* 87:3640-3644.
- Andersson S, Berman DM, Jenkins EP, Russell DW. 1991 Deletion of steroid 5 $\alpha$ -reductase 2 gene in male pseudohermaphroditism. *Nature* 354:159-161.
- Bartsch G, Klein H, Sturenburg HJ, Voigt KD. 1987 Metabolism of androgens in human benign prostatic hyperplasia: aromatase and its inhibition. *J Steroid Biochem* 27:557-664.
- Bayne CB, Donnelly F, Chapman K, Bollina P, Buck C, Habib FK. 1998 A novel coculture model for benign prostatic hyperplasia expressing both isoforms of 5 $\alpha$ -reductase. *J Clin Endocrinol Metab* 83:206-213.
- Bayne CW, Donnelly F, Ross M, Habib FK. 1999 *Serenoa repens* (Permixon): a 5 $\alpha$ -reductase types I and II inhibitor-new evidence in a coculture model of BPH. *Prostate* 40:232-41.
- Bayne CW, Ross M, Donnelly F, Habib FK. 2000 The selectivity and specificity of the actions of the lipido-sterolic extract of *Serenoa repens* (Permixon®) on the prostate. *J Urology* 164:876-81.
- Blandy JP, Lytton B. 1986 What is the prostate and what is it for? In: *The prostate*, eds. Blandy JP & Lytton B, pp. 1-11. London: Butterworths.
- Block JL, Block NL, Lokeshwar BL. 1996 Inhibition of aromatase activity and growth suppression by 4-methoxy-4-androstene-3,17-dione in an androgen sensitive human prostatic carcinoma cell line. *Cancer Letters* 101:143-148.
- Benda P, Lightbody J, Sato G, Levine L, Sweet W. 1968 Differentiated rat glial cell strain in tissue culture. *Science* 161:370-1.
- Bhatnagar AS, Hausler A, Schieweck K, Lang M, Bowman R. 1990 Highly selective inhibition of estrogen synthesis by CGS-20267, a new nonsteroidal aromatase inhibitor. *J Steroid Biochem Mol Biol* 37:1021-1027.

Bonkhoff H, Stein U, Aumuller G, Remberger K. 1996 Differential expression of 5 $\alpha$ -reductase isoenzymes in the human prostate and prostatic carcinomas. *Prostate* 29:261-267.

Bonkhoff H, Fixener T, Hunsicker I, Renberger K. 1999 Estrogen receptor expression in prostate cancer and premalignant prostatic lesions. *Am J Path* 155:641-647.

Brinkmann AO. 2001 Molecular basis of androgen insensitivity. *Mol Cell Endocrinol* 179:105-9.

Brodie AM, Son C, King DA, Meyer KM, Inkster SE. 1989 Lack of evidence of aromatase in human prostatic tissues: effects of 4-hydroxyandrostenedione and other inhibitors on androgen metabolism. *Cancer Res* 49:6551-6555.

Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER. 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med* 337:91-5.

Carilla E, Briley M, Fauran F, Sultan C, Duveilliers C. 1984 Binding of Permixon, a new treatment for prostatic benign hyperplasia, to the cytosolic androgen receptor in the rat prostate. *J Steroid Biochem* 20:521-3.

Carraro JC, Raynaud JP, Koch G, Chisholm GD, Di Silverio F, Teillac P, Da Silva FC, Cauquil J, Chopin DK, Hamdy FC, Hanus M, Hauri D, Kalinteris A, Marencak J, Perier A, Perrin P. 1996 Comparison of phytotherapy (Permixon) with finasteride in the treatment of benign prostate hyperplasia: a randomized international study of 1,098 patients. *Prostate* 29:231-40.

Chapple CR. 2000 Alpha blockade. In: *Benign prostatic hyperplasia, current therapy*, eds. Chapple CR, McConnell JD & Tubaro A, pp.1-26. London: Martin Dunitz.

Chaproniere DM, McKeehan WL. 1986 Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain. *Cancer Res.* 46:819-24.

Chevalier G, Benard P, Cousse H, Bengone T. 1997 Distribution study of radiolabelled activity in rats after oral administration of lipido/sterolic extract of *Serenoa repens* (Permixon®) supplemented with [1-<sup>14</sup>C] lauric acid, [1-<sup>14</sup>C] oleic acid or [4-<sup>14</sup>C]- $\beta$ -sitosterol. *Eur J Drug Metab Pharmacokinetics* 22:73-83.

Chomczynski P, Sacchi N. 1987 Single step method of RNA isolation by acid guanidium thiocyanate phenol chloroform extraction. *Anal Biochem* 162:156-9.

Collins AT, Zhiming B, Gilmore K, Neal DE. 1994 Androgen and oestrogen responsiveness of stromal cells derived from the human hyperplastic prostate: oestrogen regulation of the androgen receptor. *J Endocrinol* 143:269-77.



Corbin CJ, Graham-Lorence S, McPhaul M, Mason JJ, Mendelson CR and Simpson ER. 1988 Isolation of a full-length cDNA insert encoding human aromatase system cytochrome p-450 and its expression in nonsteroidogenic cells. *Proc Natl Acad USA* 85:8948-8952.

Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins S, Sugimura Y. 1987 The endocrinology and developmental biology of the prostate. *Endocrine Reviews* 8:338-362.

Daehlin L, Bergh A, Damber JE. 1987 Direct effects of oestradiol on growth and morphology of the Dunning R3327H prostatic carcinoma. *Urol Res* 15:169-72.

DeKlerk DP, Coffey DS, Ewing LL, McDermott IR, Reiner WG, Robinson CH, Scott WW, Strandberg JD, Tatalay P, Walsh PC, Wheaton LG, Zirkin BR. 1979 Comparison of spontaneous and experimentally induced canine prostatic hyperplasia. *J Clin Invest* 64:842-849.

Délos S, Carsol JL, Ghazarossian E, Raynaud JP, Martin PM. 1995 Testosterone metabolism in primary cultures of human prostate epithelial cells and fibroblasts. *J Steroid Biochem Mol Biol* 55:375-383.

Délos S, Carsol JL, Fina F, Raynaud JP, Martin PM. 1998 5 $\alpha$ -reductase and 17 $\beta$ -hydroxysteroid dehydrogenase expression in epithelial cells from hyperplastic and malignant human prostate. *Int J Cancer* 75:840-846.

De Wet JR, Wood KV, De Luca M, Helinski DR, Subramani S. 1987 Firefly luciferase gene – structure and expression in mammalian cells. *Mol Cell Biol* 7:725-737.

Di Silverio F, D'Eramo G, Lubrano C, Flammia GP, Sciarra A, Palma E, Caponera M, Sciarra F. 1992 Evidence that *Serenoa repens* extract display an antiestrogenic activity in prostatic tissue of benign prostatic hypertrophy patients. *European Urology* 21:309-314.

Di Silverio F, Monti S, Sciarra A, Varasano PA, Martini C, Lanzara S, D'Eramo G, Di Nicola S, Toscano V. 1998 Effects of long-term treatment with *Serenoa repens* (Permixon®) on the concentrations and regional distribution of androgens and epidermal growth factor in benign prostatic hyperplasia. *Prostate* 37:77-83.

Djavan B, Marberger M. 1999 A meta-analysis on the efficacy and tolerability of alpha1-adrenoceptor antagonists in patients with lower urinary tract symptoms suggestive of benign prostatic obstruction. *Eur Urol* 36:1-13.

Ehara H, Koji T, Deguchi T, Yoshii A, Nakano M, Nakane PK, Kawada Y. 1995 Expression of estrogen receptor in diseased human prostate assessed by non-radioactive in situ hybridization and immunohistochemistry. *Prostate* 27:304-13.

- Eicheler W, Tuohimaa P, Vilja P, Adermann K, Forssmann WG, Aumuller G. 1994 Immunocytochemical localization of human 5  $\alpha$ -reductase 2 with polyclonal antibodies in androgen target and non-target human tissues. *J Histochem Cytochem* 42:667-75.
- El-Alfy M, Luu-The V, Huang XF, Berger L, Labrie F, Pelletier G. 1999 Localization of type 5 17 $\beta$ -hydroxysteroid dehydrogenase, 3 $\beta$ -hydroxysteroid dehydrogenase, and androgen receptor in the human prostate by in situ hybridization and immunocytochemistry. *Endocrinol* 140:1481-91.
- Elo JP, Akinola LA, Poutanen M, Vihko P, Kyllonen AP, Lukkarinen O, Vihko R. 1996 Characterization of 17 $\beta$ -hydroxysteroid dehydrogenase isoenzyme expression in benign and malignant human prostate. *Int J Cancer* 66:37-41.
- Enmark E, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M, Gustafsson JA. 1997 Human estrogen receptor  $\beta$ -gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 82:4258-65.
- Evans RM. 1988 The steroid and thyroid hormone receptor superfamily. *Science* 240:889-95.
- Farnsworth WE. 1999 Estrogen in the etiopathogenesis of BPH. *Prostate* 41:263-74.
- Fisher CR, Graves KH, Parlow AF, Simpson ER. 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci USA* 95:6965-70.
- Ghanadian R, Puah CM. 1981 Relationships between oestradiol-17 $\beta$ , testosterone, dihydrotestosterone and 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol in human benign hypertrophy and carcinoma of the prostate. *J Endocrinol* 88:255-262.
- Gluzman Y. 1981 SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* 23:175-82.
- Grant ES, Batchelor KW, Habib FK. 1996 Androgen independence of primary epithelial cultures of the prostate is associated with a down-regulation of androgen receptor gene expression. *Prostate* 29:339-49.
- Gray A, Feldman HA, McKinlay JB, Longcope C. 1991 Age, disease and changing sex hormone levels in middle-aged men: results of the Massachusetts male aging study. *J Clin Endocrinol Metab* 73:1016-1025.
- Green S, Chambon P. 1986 A superfamily of potentially oncogenic hormone receptors. *Nature* 324:615-7.

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. 1986 Sequence and expression of human estrogen receptor complementary DNA. *Science* 231:1150-4.

Griffin JE, Wilson JD. 1984 Disorders of androgen receptor function. *Ann NY Acad Sci* 438:61-71.

Griffin JE, Wilson JD. 1992 Disorders of the testis and the male reproductive tract. In: Williams textbook of endocrinology (8<sup>th</sup> ed.), eds. Wilson JD & Foster DW, pp. 799-852. Philadelphia: W.B. Saunders.

Guess HA. 1995 The prevalence of benign prostatic hyperplasia in population surveys. In: Epidemiology of prostate disease, ed. Garraway M. pp.121-131. Berlin: Springer Verlag.

Habib FK, Lee IR, Stitch SR, Smith PH. 1976 Androgen levels in the plasma and prostatic tissues of patients with benign hypertrophy and carcinoma of the prostate. *J Endocrinol* 71:99-107.

Habib FK. 1992 Regulation of prostate growth in culture with the pollen extract, cernitin T-60 and the impact of the drug on the EGF tissue profiles. In: Benign prostate diseases, eds Vahlensieck W, Rutishauser G, pp. 115-122. Stuttgart, Thieme Verlag,

Habib FK. 1994 Pathogenesis of benign prostatic hyperplasia. In: Handbook on benign prostatic hyperplasia, ed. Chisholm GD, pp. 19-31. New York: Raven Press.

Habib FK, Ross M, Bayne CW, Grigor K, Buck AC, Bollina P, Chapman K. 1998 The localisation and expression of 5 $\alpha$ -reductase types I and II mRNAs in human hyperplastic prostate and in prostate primary cultures. *J Endocrinol* 156:509-517.

Harada N, Utsumi T, Takagi Y. 1993 Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. *Proc Natl Acad Sci U S A* 90:11312-11316.

Harada N. 1999 Aromatase and intracrinology of estrogen in hormone-dependent tumors. *Oncology* 57 Suppl 2:7-16.

Hayward SW, Dahiya R, Cunha GR, Bartek J, Deshpande N, Narayan P. 1995 Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell Dev Biol Anim* 31:14-24.

Helpap B. 1992 Patholgy of benign prostatic hyperplasia. In: Benign prostate diseases, eds. Vahlensieck W, Rutishauser G, pp. 84-97. Stuttgart, Thieme Verlag,

Hillier SG, van den Boogaard AMJ, Reichert LE, van Hall EV. 1980 Alterations in granulosa cell aromatase activity accompanying preovulatory follicular development in the rat ovary with evidence that  $5\alpha$ -reduced C19 steroids inhibit the aromatase reaction in vitro. *J Endocrinol* 84:409-419.

Hiramatsu M, Maehara I, Ozaki M, Harada N, Orikasa S, Sasano H. 1997 Aromatase in hyperplasia and carcinoma of the human prostate. *Prostate* 31:118-124.

Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP. 1983 LNCaP model of human prostatic carcinoma. *Cancer Res* 43:1809-18.

Houston B, Chisholm GD, Habib FK. 1985 Solubilization of human prostatic  $5\alpha$ -reductase. *J Steroid Biochem* 22:461-7.

Hudson RW, Wherrett D. 1990 Comparison of the  $5\alpha$ -reduction of testosterone and androstenedione in human prostatic carcinoma and benign prostatic hyperplasia. *J Steroid Biochem* 35:231-236.

Iehle C, Delos S, Guirou O, Tate R, Raynaud JP, Martin PM. 1995 Human prostatic steroid  $5\alpha$ -reductase isoforms--a comparative study of selective inhibitors. *J Steroid Biochem Mol Biol* 54:273-9.

Iehle C, Radvanyi F, Gil Diez de Medina S, Ouafik LH, Gerard H, Chopin D, Raynaud JP, Martin PM. 1999 Differences in steroid  $5\alpha$ -reductase iso-enzymes expression between normal and pathological human prostate tissue. *J Steroid Biochem Mol Biol* 68:189-95.

Imperato-McGinley J, Peterson RE, Gautier T 1981 Male pseudohermaphroditism secondary to  $5\alpha$ -reductase deficiency: a review. In: *Fetal Endocrinology*, eds. Novy MJ & Resko JA, pp. 359-382. New York: Academic Press.

Imperato-McGinley J, Gautier T, Zirinsky K, Hom T, Palomo O, Stein E, Vaughan ED, Markisz JA, Ramirez de Arellano E, Kazam E. 1992 Prostate visualization studies in males homozygous and heterozygous for  $5\alpha$ -reductase deficiency. *J Clin Endocrinol Metab* 75:1022-1026.

Isaacs JT, Coffey DS. 1989 Etiology and disease process of benign prostatic hyperplasia. *Prostate Suppl.* 2:33-50.

Jenkins EP, Hsieh CL, Milatovich A, Normington K, Berman DM, Francke U, Russell DW. 1991 Characterization and chromosomal mapping of a human steroid  $5\alpha$ -reductase gene and pseudogene and mapping of the mouse homologue. *Genomics* 11:1102-1112.

- Jenkins EP, Andersson S, Imperato-McGinley J, Wilson J, Russell DW. 1992 Genetic and pharmacological evidence for more than one human steroid 5 $\alpha$ -reductase. *J Clin Invest* 89:293-300.
- Kaburagi Y, Marino MB, Kirdani RY, Greco JP, Karr JP, Sandberg AA. 1987 The possibility of aromatization of androgen in human prostate. *J Steroid Biochem* 26:739-742.
- Kaefer M, Audia JE, Bruchovsky N, Goode RL, Hsiao KC, Leibovitch IY, Krushinski JH, Lee C, Steidle CP, Sutkowski DM, Neubauer BL. 1996 Characterization of type I 5  $\alpha$ -reductase activity in DU145 human prostatic adenocarcinoma cells. *J Steroid Biochem Mol Biol* 58:195-205.
- Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. 1979 Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 17:16-23.
- Katz MD, Cai LQ, Zhu YS, Herrera C, DeFillo-Ricart M, Shackleton CH, Imperato-McGinley J. 1995 The biochemical and phenotypic characterization of females homozygous for 5 $\alpha$ -reductase-2 deficiency. *J Clin Endocrinol Metab* 80:3160-3167.
- Katzenellenbogen BS & Korach KS. 1997 A new actor in the estrogen receptor drama – enter ER $\beta$ . *Endocrinol* 138:861-862.
- Katzenellenbogen BS, Montano M, Ediger TR, Sun J, Ekena K, Martini PG, McInerney EM, Delage-Mourroux R, Weis K, Katzenellenbogen JA. 2000 Estrogen receptors: selective ligands, partners and distinctive pharmacology. *Recent Progress Hormone Research* 55:163-195.
- Klinge CM. 2000 Estrogen receptor interaction with co-activators and co-repressors. *Steroids* 65:227-51.
- Krazeisen A, Breitling R, Imai K, Fritz S, Moller G, Adamski J. 1999 Determination of cDNA, gene structure and chromosomal localization of the novel human 17 $\beta$ -hydroxysteroid dehydrogenase type 7. *FEBS Lett.* 460:373-9.
- Krekels MD, Wouters W, De Coster R, Van Ginckel R, Leonaers A, Janssen PA. 1991 Aromatase in the human choriocarcinoma JEG-3: inhibition by R 76 713 in cultured cells and in tumors grown in nude mice. *J Steroid Biochem Mol Biol* 38:415-22.
- Krieg M, Weissner H, Tunn S. 1995 Potential activities of androgen metabolising enzymes in human prostate. *J Steroid Biochem Mol Biol* 53:395-400.
- Krust A, Green S, Argos P, Kumar V, Walter P, Bornert JM, Chambon P. 1986 The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO J* 5:891-7.



Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA. 1996 Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93:5925-30.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinol* 138:863-70.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinol* 139:4252-63.

Labrie F, Luu-The V, Lin SX, Labrie C, Simard J, Breton R, Belanger A. 1997 The key role of 17 beta-hydroxysteroid dehydrogenases in sex steroid biology. *Steroids* 62:148-58.

Labrie F, Luu-The V, Lin SX, Simard J, Labrie C, El-Alfy M, Pelletier G, Belanger A. 2000 Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease. *J Mol Endocrinol* 25:1-16.

Lau KM, Mok SC, Ho SM. 1999 Expression of human estrogen receptor-alpha and -beta, progesterone receptor, and androgen receptor mRNA in normal and malignant ovarian epithelial cells. *Proc Natl Acad Sci USA* 96:5722-7.

Lau KM, LaSpina M, Long J, Ho SM. 2000 Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. *Cancer Res* 60:3175-82.

Levine AC, Ren M, Huber GK, Kirschenbaum A. 1992 The effect of androgen, estrogen, and growth factors on the proliferation of cultured fibroblasts derived from human fetal and adult prostates. *Endocrinol* 130:2413-2419.

Levy MA, Brandt M, Sheedy KM, Holt DA, Heaslip JJ, Trill JJ, Ryan PJ, Morris RA, Garrison LM & Bergsma DL. 1995 Cloning, expression and functional characterization of type 1 and type 2 steroid 5 $\alpha$ -reductases from *Cynomolgus* monkey: comparisons with human and rat isoenzymes. *J Steroid Biochem Mol Biol* 52:307-319.

Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, Huhtaniemi I, Santti R, Makela S, Poutanen M. 2001 Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. *Endocrinol* 142:2435-42.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275.

MacGregor JJ, Jordan VC. 1998 Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev* 50:151-96.

- Maghni K, Nicolescu OM, Martin JG. 1999 Suitability of cell metabolic colorimetric assays for assessment of CD4+ T cell proliferation: comparison to 5-bromo-2-deoxyuridine (BrdU) ELISA. *J Immunol Methods* 223:185-94.
- Mahendroo MS, Cala KM, Russell DW. 1996 5 $\alpha$ -reduced androgens play a key role in murine parturition. *Mol Endocrinol* 10:380-92.
- Mahendroo MS, Cala KM, Landrum CP, Russell DW. 1997 Fetal death in mice lacking 5 $\alpha$ -reductase type 1 caused by estrogen excess. *Mol Endocrinol* 11:917-927.
- Mahendroo MS, Cala KM, Hess DL, Russell DW. 2001 Unexpected virilization in male mice lacking steroid 5 $\alpha$ -reductase enzymes. *Endocrinol* 142:4652-62.
- Matzkin H, Soloway MS. 1992 Immunohistochemical evidence of the existence and localization of aromatase in human prostatic tissues. *Prostate* 21:309-314.
- McNeal JE. 1968 Regional morphology and pathology of the prostate. *Am J Clin Pathol* 49:347-57.
- McNeal JE. 1978 Origin and evolution of benign prostatic enlargement. *Invest Urol* 15:340-5.
- McNeal JE. 1981 The zonal anatomy of the prostate. *Prostate* 2:35-49.
- McNeal JE. 1989 Anatomy and embryology. In: The prostate, eds. Fitzpatrick JM, Krane RJ, pp. 3-9. Edinburgh: Churchill Livingstone.
- McNeal JE. 1990 The pathobiology of nodular hyperplasia. In: Pathology of the prostate, ed. Bostwick DG. New York: Churchill Livingstone.
- McPherson SJ, Wang H, Jones ME, Pedersen J, Iismaa TP, Wreford N, Simpson ER, Risbridger GP. 2001 Elevated androgens and prolactin in aromatase-deficient mice cause enlargement, but not malignancy, of the prostate gland. *Endocrinol* 142:2458-2467.
- Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J Clin Endocrinol Metab* 80:3689-3698.
- Morita K, Tsuruo Y, Ishimura K, Her S, Bell RA, Wong DL. 1999 Influence of serum-free culture conditions on steroid 5 $\alpha$ -reductase mRNA expression in rat C6 glioma cells. *Brain Res* 830:179-82.
- Mosselman S, Polman J, Dijkema R. 1996 ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 392:49-53.

- Negri-Cesi P, Poletti A, Colciago A, Magni P, Martini P, Motta M. 1998 Presence of 5 $\alpha$ -reductase isozymes and aromatase in human prostate cancer cells and in benign prostate hyperplastic tissue. *Prostate* 34:283-291.
- Negri-Cesi P, Colciago A, Poletti A, Motta M. 1999 5 $\alpha$ -reductase isozymes and aromatase are differentially expressed and active in the androgen-independent human prostate cancer cell lines DU145 and PC3. *Prostate* 41:224-32.
- Neuzil E, Cousse H. 1993 *Serenoa repens*, aspects botaniques et chimiques. *Bull Soc Pharm Bordeaux* 132:121-141.
- Nordling E, Oppermann UC, Jornvall H, Persson B. 2001 Human type 10 17  $\beta$ -hydroxysteroid dehydrogenase: molecular modelling and substrate docking. *J Mol Graph Model* 19:514-20, 591-3.
- Oesterling JE, Epstein JI, Walsh PC. 1986 The inability of adrenal androgens to stimulate the adult human prostate: an autopsy evaluation of men with hypogonadotropic hypogonadism and panhypopituitarism. *J Urol* 136:1030-4.
- Orth DN, Kovacs, Debold CR. 1992 The adrenal cortex. In: Williams textbook of endocrinology (8<sup>th</sup> ed.), eds. Wilson JD, Foster DW, pp. 489-620. Philadelphia: W.B. Saunders.
- Pardridge WM. 1981 Transport of protein-bound hormones into tissues in vivo. *Endocr Reviews* 2:103-23.
- Pasquali D, Staibano S, Prezioso D, Franco R, Esposito D, Notaro A, DeRosa G, Bellastella A, Sinisi AA. 2001a Estrogen receptor beta expression in human prostate tissue. *Mol Cell Endocrinol* 178:47-50.
- Pasquali D, Rossi V, Esposito D, Abbondanza C, Puca GA, Bellastella A, Sinisi AA. 2001b Loss of estrogen receptor beta expression in malignant human prostate cells in primary cultures and in prostate cancer tissues. *J Clin Endocrinol Metab* 86:2051-5.
- Peltoketo H, Luu-The V, Simard J, Adamski J. 1999 17 $\beta$ -hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J Mol Endocrinol* 23:1-11.
- Penning TM. 1997 Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocrine Rev* 18:281-305.
- Perel E, Stolee KH, Kharlip L, Blackstein ME, Killinger DW. 1984 The intracellular control of aromatase activity by 5 $\alpha$ -reduced androgens in human breast carcinoma cells in culture. *J Clin Endocrinol Metab* 58:467-472.
- Plosker GL, Brogden RN. 1996 *Serenoa repens* (Permixon), a review of its pharmacology and therapeutic efficacy in benign prostatic hyperplasia. *Drugs Aging* 9:379-95.



Promega Protocols and Applications Guide. 1996 (3<sup>rd</sup> ed.), ed. Doyle K. USA

Puck TT. 1958 Genetics of somatic mammalian cells III. Long-term cultivation of euploid cells from human and animal subjects. *J Exp Med* 108: 945-956.

Pylkkanen L, Santti R, Salo L, Maentausta O, Vihko R, Nurmi M. 1994 Immunohistochemical localization of estrogen-specific 17 $\beta$ -hydroxysteroid oxidoreductase in the human and mouse prostate. *Prostate* 25:292-300.

Reed KC, Ohno S. 1976 Kinetic properties of human placental aromatase; application of an assay measuring <sup>3</sup>H<sub>2</sub>O release from 1 $\beta$ ,2 $\beta$ -<sup>3</sup>H-androgens. *J Biol Chem* 251:1625-31.

Rittmaster RS. 1994 Finasteride. *New Engl J Med* 330:120-124.

Robinson EJ, Neal DE, Collins AT. 1998 Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium. *Prostate* 37:149-60.

Royuela M, De Miguel MP, Bethencourt FR, Sanchez-Chapado M, Fraile B, Arenas MI, Paniagua R. 2001 Estrogen receptors  $\alpha$  and  $\beta$  in the normal, hyperplastic and carcinomatous human prostate. *J Endocrinol* 168:447-454.

Russell DW, Wilson JD. 1994 Steroid 5 $\alpha$ -reductase: two genes/two enzymes. *Ann Rev Biochem* 63:25-61.

Sambrook J, Fritsch EF, Maniatis T. 1989 Molecular cloning: a laboratory manual. (2<sup>nd</sup> ed). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sasano H, Harada N. 1998 Intratumoral aromatase in human breast, endometrial, and ovarian malignancies. *Endocrine Rev* 19:593-607.

Saunders PT. 1998 Oestrogen receptor beta (ER $\beta$ ). *Rev Reprod* 3:164-71.

Schweikert HU. 1979 Conversion of androstenedione to estrone in human fibroblasts cultured from prostate, genital and nongenital skin. *Horm Metab Res* 11:635-640.

Sebastian S, Bulun SE. 2001 A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project. *J Clin Endocrinol Metab* 86:4600-2.

Sharpe RM. 2001 Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicology Letters* 120:221-32.

Siiteri PK, Wilson JD. 1970 Dihydrotestosterone in prostatic hypertrophy I, the formation and content of dihydrotestosterone in the hypertrophic prostate of man. *J Clin Invest* 49:1737-45.

Silver RI, Wiley EL, Thigpen AE, Guileyardo JM, McConnell JD, Russell DW. 1994 Cell type specific expression of steroid 5 $\alpha$ -reductase 2. *J Urol* 152:438-442.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR, Bulun SE. 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Rev* 15:342-355.

Simpson ER, Zhao Y, Agarwal VR, Michael MD, Bulun SE, Hinshelwood MM, Graham-Lorence S, Sun T, Fisher CR, Qin K, Mendelson CR. 1997 Aromatase expression in health and disease. *Recent Progress Hormone Research* 52:185-213.

Smith T, Chisholm GD, Habib FK. 1982 Failure of human benign prostatic hyperplasia to aromatise testosterone. *J Steroid Biochem* 17:119-120.

Smith CM, Ballard SA, Worman N, Buettner R, Masters JRW. 1996 5 $\alpha$ -reductase expression by prostate cancer cell lines and benign prostatic hyperplasia in vitro. *J Clin Endocrinol Metab* 81:1361-1366.

Stone KR, Mickey DD, Wunderli H, Mickey G, Paulson D. 1978 Isolation of a human prostate carcinoma cell line (DU145). *Int J Cancer* 21:274-281.

Stone NN, Fair WR, Fishman J. 1986 Estrogen formation in human prostatic tissue from patients with and without benign prostatic hyperplasia. *Prostate* 9:311-318.

Stone NN, Laudone VP, Fair WR, Fishman J. 1987 Aromatization of androstenedione to estrogen by benign prostatic hyperplasia, prostate cancer and expressed prostatic secretions. *Urol Res* 15:165-167.

Stone NN, Clejan SJ. 1991 Response of prostate volume, prostate-specific antigen, and testosterone to flutamide in men with benign prostatic hyperplasia. *J Androl* 12:376-80.

Sugimura Y, Cunha GR, Yonemura CU, Kawamura J. 1988 Temporal and spatial factors in diethylstilboestrol-induced squamous metaplasia of the developing human prostate. *Hum Pathol* 19:133-139.

Suzuki T, Sasano H, Andersson S, Mason JJ. 2000 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow \Delta^4$ -isomerase activity associated with the human 17 $\beta$ -hydroxysteroid dehydrogenase type 2 isoform. *J Clin Endocrinol Metab* 85:3669-72.

Thigpen AE, Davis DL, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, Francke U, Wilson JD, Russell DW. 1992 Molecular genetics of steroid 5  $\alpha$ -reductase 2 deficiency. *J Clin Invest* 90:799-809.

- Thigpen AE, Cala KM & Russell DW. 1993a Characterization of Chinese hamster ovary cell lines expressing human steroid 5 $\alpha$ -reductase isozymes. *J Biol Chem* 268:17404-17412.
- Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD, Russell DW 1993b Tissue distribution and ontogeny of steroid 5 $\alpha$ -reductase isozyme expression. *J Clin Invest* 92:903-910.
- Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* 11:353-65.
- Tsugaya M, Habib FK, Chisholm GD, Ross M, Tozawa K, Hayashi Y, Kohri K, Tanaka S. 1996 Testosterone metabolism in primary cultures of epithelial cells and stroma from benign prostatic hyperplasia. *Urol Res* 24:265-271.
- Turcotte G, Chapdelaine A, Roberts KD, Chevalier S. 1988 Androgen binding as evidenced by a whole cell assay system using cultured canine prostatic epithelial cells. *J Steroid Biochem* 29:69-76.
- Tutrone RF Jr, Ball RA, Ornitz DM, Leder P, Richie JP. 1993 Benign prostatic hyperplasia in a transgenic mouse: a new hormonally sensitive investigatory model. *J Urol* 149:633-9.
- Uson AC, Paez AB, Uson-Jaeger J. 1991 The natural history and course of untreated benign prostatic hyperplasia. *Eur Urol* 20(suppl):22-26.
- Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E. 1992 The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J Steroid Biochem Mol Biol* 41:665-9.
- Voigt KD, Bartsch W. 1986 Intratissular androgens in benign prostatic hyperplasia and prostatic cancer. *J Steroid Biochem* 25:749-757.
- Walsh PC, Madden JD, Harrod MJ, Goldstein JL, MacDonald PC, Wilson JD. 1974 Familial incomplete male pseudohermaphroditism, type 2. *New Engl J Med* 291:944-949.
- Walsh PC, Wilson JD. 1976 The induction of prostatic hypertrophy in the dog with androstanediol. *J Clin Invest* 57:1093-1097.
- Walsh PC, Hutchins GM, Ewing LL. 1983 Tissue content of dihydrotestosterone in human prostatic hyperplasia is not supranormal. *J Clin Invest* 72:1772-1777.
- Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster JJ, Jensen EV, Nilsson S, Warner M, Gustafsson JA. 2001 A role for estrogen receptor beta in the regulation of growth of the ventral prostate. *Proc Natl Acad Sci USA* 98:6330-5.

Williams GR, Franklyn JA. 1994 Physiology of the steroid-thyroid hormone nuclear receptor superfamily. *Bailliere's Clin Endocrinol Metab* 8:241-266

Wilt TJ, Ishani A, Stark G, MacDonald R, Lau J, Mulrow C. 1998 Saw palmetto extracts for treatment of benign prostatic hyperplasia: a systematic review. *JAMA* 280:1604-9.

Wilt TJ. 2000 Phytotherapy. In: *Benign prostatic hyperplasia: current therapy*, eds. Chapple CR, McConnell JD & Tubaro A, pp. 39-56. London: Martin Dunitz

Wu L, Einstein M, Geissler WM, Chan HK, Elliston KO, Andersson S. Expression cloning and characterization of human 17 beta-hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20alpha-hydroxysteroid dehydrogenase activity. *J Biol Chem* 268:12964-12969.

Zhu YS, Katz MD, Imperato-McGinley J. 1998 Natural potent androgens: lessons from human genetic models. *Bailliere's Clinical Endocrinol Metab* 12:83-113.

## PUBLICATIONS FROM THIS THESIS

Ho C.K.M., Martin C., Andrew R., Bollina P.R., Chapman K.E., Habib F.K. Regulation of aromatase activity by 5 $\alpha$ -reductase type 1 isozyme in the human prostate. Submitted to Journal of Endocrinology.

Ho C.K.M., Martin C., Andrew R., Chapman K.E., Habib F.K. Effects of the lipidosterolic extract of *Serenoa repens* (Permixon<sup>®</sup>) on formation and catabolism of oestrogens in the human prostate. In preparation.